Cellular Interactions between Host and Parasite

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I declare that this thesis was composed by myself and the work described therein to be my own

Adrian D. Schofield

Abstract

This thesis describes a research project which investigated the cellular interactions between the root-less twining stem parasite, Cuscuta campestris (Convulvulaceae) and the herbaceous plant, Pelargonium zonale (Geraniaceae).

This parasite is a member of a genus of Angiosperm plants which gain nutrients from host plants by coiling around and penetrating the shoots with absorptive feeding organs. Cuscuta campestris invades a wide range of plant hosts and is able to do so without apparently invoking any resistance response. A particular interest in this aspect of its biology was the reason for the work described here.

A description of the structural and morphological changes which occur during invasion, recorded employing Light, Scanning Electron and Transmission Electron Microscopy, establishes the main events which take place when Pelargonium plants are parasitised by Cuscuta. It also provides important clues as to the underlying biochemical changes.

Much of the work described concerns the successful development of a novel in vitro technique, which allows a measure of manipulation of the host/parasite interaction. Sterile tissues of host and parasite are brought into contact in a controlled environment. During the development of this in vitro technique strenuous attempts were made to remove variation due to genomic differences, differing physiological states and the fluctuating physical and chemical environment. Experiments are described which assess the effect of specific, physical and environmental factors on the ability of the parasite to infect. Development also included the devising of culture and inoculation procedures and a verification that the interaction in vitro was representative of that in vivo, by comparing structural records of events.

Results from an investigation into the role of the cell wall degrading enzymes, cellulase, pectin esterase and polygalacturonase in the parasitism of Pelargonium plants by Cuscuta, suggest that cellulase, at least is of considerable importance. During this investigation it was considered essential to monitor changes in the activities of the enzymes during the time course of infection and to relate any changes to the structural and morphological changes in order to determine if they had a specific role in parasitism by Cuscuta.

In the discussion particular attention is given to the way Cuscuta has adapted to the parasitic habit, its success as a parasite and the usefulness of the in vitro technique developed. Possible mechanisms which may be used by Cuscuta to avoid invoking resistance in host plants are suggested.

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Abbreviations

ABBREVIATIONS

Substances

chl	chl[illegible]hyll
IAA	indole acetic acid
GA_3	gibberelic acid
2,4 D	2,4, dichlorophenoxy acetic acid
CMC	carboxy methyl cellulose
PGURA	polygalacturonic acid
M and S	Murashige and Skoog plant growth medium
BM	basal medium
BMGA	basal medium with gibberelic acid (1.10^{-4} M)
chla	chlorophyll a
chlb	chlorophyll b

Enzymes

CE	cellulase
PGURASE	polygalacturonase
PE	pectin esterase

Microscopy

LM	light microscopy
SEM	scanning electron microscopy
TEM	transmission electron micropscopy
EM	electron microscopy

Units

mm	millimetre
cm	centimetre
m	metre
s	second

Abbreviations contd

min	minute
hr	hour
µl	microlitre
ml	millilitre
l	litre
µg	microgram
mg	milligram
g	gram
M	molar

Statistical

se	standard error
sd	standard deviation

Other

TS	transverse section
Ch	chapter
RM	reaction mixture

Chapter 1

Chapter 1 - THE INTRODUCTION

The study of cellular interactions between host and parasite is an area of active research in biology. Perhaps the main reason for this is that parasites are responsible for causing extensive crop damage, and it is therefore essential to find ways of reducing the resulting huge financial losses. When host plants are invaded by a parasite, groups of cells often act together in a co-ordinated way to produce a defensive response e.g. in hypersensitive reactions (Kiraly and Barna 1986), in the formation of lignin barriers (Vance *et al.* 1980) and during the production of antibiotic chemicals (Bailey and Mansfield 1982). Therefore, an understanding of the mechanisms involved in these interactions will not only help to eliminate or alleviate crop disease, but tell us more about the nature of cell/cell communications in general.

The parasitic habit

Parasitism as a lifestyle is not confined to one or a few types of organism. Viroids, viruses, bacteria and fungi can all be pathogens of higher plants, remaining in contact with their hosts for prolonged periods. Viroids and viruses cannot technically be said to undergo cellular interactions with a host plant because they are subcellular organisms. Within each group of organisms there are major differences between genera and often between species. This is shown in particular with fungi. Some species invade the host only to a limited extent, others penetrate the host, but only enter the intercellular spaces, while yet others actually penetrate the cells of the host. The spectrum of different levels of involvement and the different interfaces involved are complex (see review Bracker and Littlefield 1973), emphasising that care should always be exercised when making generalisations about parasites and their interelationships with the host.

Parasitic Angiosperms

The parasite chosen for the work embodied in this thesis is a member of another, perhaps less familiar group of organisms, the parasitic Angiosperms. There are at least eight unrelated groups of higher plants which have become adapted, apparently independently to the parasitic habit (Kuijt 1983). Although unrelated they have one characteristic in common; they all posess a specialised organ which penetrates the living tissues of the host in order to absorb nutrients. The nature of these organs or 'haustoria' varies greatly between the different groups, but all are usually found to form intimate contact with the vascular tissues of the host and withdraw nutrients from the phloem, xylem or both. In forming these intimate cellular contacts with host tissues the Angiosperm parasites appear to have overcome the problems of rejection which arise when unrelated plant species are grafted together (a heterograft). The behaviour of these

parasites presents a sharp contrast with that of a stock and scion (partners in a graft), where it is found that functional vascular connections tend only to form between partners with a close taxanomic relationship (Yeoman 1984). Indeed it has been shown in the Leguminosae that the ability to graft can be used as an indicator of the closeness of the taxanomic relationship between members of the family (Kloz 1971). The cellular relationships that exist between an Angiosperm parasite and its host appear to parallel the heterograft, in that it involves cellular interaction between two unrelated higher plants. However, Angiosperm parasites also have much, if not more in common with the invasive fungi. For example both Angiosperm and fungal parasites have developed mechanisms for penetrating a host, for overcoming host resistance and for absorbing nutrients. Indeed the desire to understand how an Angiosperm parasite overcomes the resistance of the host plant was the major reason for undertaking the work reported in this thesis, as it is not common for interaction between cells of different species to be compatible.

The responses of higher plants to contact with cells of other species

The Interfaces between two different organisms often show signs of incompatibility. When unrelated plants are grafted together necrotic regions may develop at the interface between the two plants. This was found by Moore and Walker (1981) in grafts between plants of *Sedum* and *Solanum* and by Fuji and Nito (1972) when grafting together callus derived from different species.

Higher plants have been found to respond to invasion by parasites in several different ways. Hypersensitivity, involving rapid necrosis of host cells at the penetration site is a common response (Kiraly and Bonna 1986). Here the death of host cells is thought to restrict the invading organism and disrupt the supply of nutrients to it (Maclean *et al.* 1971). Many plants form lignified barriers in response to invasion by parasites (Vance *et al* . 1980, Ride and Pearce 1979). The lignin laid down in the walls of individual or groups of cells is thought to increase the mechanical resistance of plant cells to compressive forces generated by the advancing hyphae. More localised thickenings develop in some plant cells but these are not necessarily of lignin and have been described as papillae (see Aist 1976). As well as stimulating physical changes, invasion by a parasite can induce the production of antibiotic substances. These may take the form of enzymes which resist the parasite by degrading the invading organ. For example plants may in certain circumstances produce chitinase which will degrade fungal cell walls (Abeles *et al.* 1978), and proteolytic enzymes capable of degrading the outer coat of virus particles (Koenig *et al.* 1978). However, a more common response to invasion is the production of substances toxic to the parasite, named phytoalexins (Bailey and Mansfield 1982). There are many different kinds of phytoalexin , including isoflavonoids, flavonoids,

dihydrophenanthrenes, stilbenes, coumarins, isocoumarins, terpenoids, furanacetylenes, polyacetylenes and polyenes. Some, such as phaseollin, pisatin, rishitin and phytotuberin are also toxic to the host, disrupting plasma membranes and interfering with mitochondrial activity. However, the host plants which produce these substances overcome the problem by possessing the means to detoxify them (Chapra *et al.* 1974, Yoshikawa *et al.* 1979). As plants have evolved mechanisms for combatting invasion so parasites have evolved mechanisms for overcoming resistance.

Overcoming host resistance

Bell (1981) divided parasites into three main groups.

(1) Parasites which do not stimulate any of the standard resistance responses (see above).

(2) Parasites which cause host resistance to be triggered after a short delay.

(3) Parasites which readily trigger host resistance.

Organisms in group one are thought to avoid host resistance by producing something which suppresses their detection or by failing to produce substances which normally betray their presence. The short lived resistance of organisms in group (2) may operate using similar mechanisms. Organisms from group (3) may not have any mechanisms for overcoming host resistance or those which they do possess are ineffective. However, the absence of a mechanism for overcoming host resistance does not mean that an organism cannot be a successful parasite but it may determine the level of intimacy which can be achieved between parasite and host. Accordingly an organism which penetrates host tissues and remains in close contact with them for prolonged periods, is more likely to have a complex mechanism for overcoming or preventing host resistance than one which does not penetrate the host to gain nutrients but disrupts the tissues enzymatically. As Angoisperm parasites spend prolonged periods in close contact with their hosts it is probable that they lie between groups (1) and (2). Indeed in many instances they are able to penetrate the tissues of the host without apparently stimulating any response from the host. However this is not always the case. Tsivion (1979) showed that a certain variety of *Lycopersicon esculentum* was able to express resistance to invasion by the twining stem parasite *Cuscuta campestris*.

The experimental approach.

Clearly a study of the morphological, structural and biochemical changes which take place as a parasite becomes established on a host plant is an essential prerequisite to obtaining an understanding of the interaction between the two organisms. Such studies must be carried out under defined standard conditions in order to reduce variability. Research on other host/parasite interactions has shown which factors are likely to generate variability in host/ parasite interactions. Some of the factors which influence the

response of the host to invasion by a parasite are as follows:-

(1)The ability to respond to an invading parasite can be genetically controlled (Day 1974, Nelson 1973 and Russel 1979). For example cultivars within a species may show a wide variation in their level of resistance to any given pathogen. In order to achieve uniformity it was decided to use cloned material of both host and parasite.

(2) Environmental factors have been shown to influence the ability of higher plants to respond to an invading parasite. For example temperature was found to affect the expression of resistance in certain cotton cultivars. Resistance to *Verticillium* was expressed at 29°C but not at 25°C (Bell and Presley 1969). In contrast to this the wheat gene Sr6 was found to confer resistance to *Puccinia graminis* at 20°C but not at 25°C (Mayama et al. 1975). Light intensity and duration have also been found to influence the expression of resistance. Depending on the host/parasite combination low light intensity may increase or decrease the resistance response (Hammerschmidt and Nicolson 1977 and Benedict 1972). So that in order to reduce the variation in the response of host plants to invasion by a parasite in a particular experiment, it is important to be able to control the light and temperature. This can be partially achieved by placing infected host plants inside growth cabinets or rooms in which the environment is controlled. However, if the host plants are large it is difficult to maintain identical conditions all over their surfaces.

(3)The physiological age and nutritional conditions of a plant can affect its response to invasion by a parasite. In a certain tomato variety only plants which were 15 days or older were able to respond hypersensitively to infection by *Cuscuta campestris* (Tsivion 1979). Younger plants showed no such reaction. The mineral nutrition of plants has also been found to influence the ability of the plant to respond to pathogens (International Potash Institute 1976). For these reasons it was considered important to use host plants of the same physiological age under the same nutritional conditions.

(4) Another factor which is likely to cause some variation in the response of the host to the primary infection is a secondary infection by microorganisms. Microorganisms may become established in wounded tissues produced by the Angiosperm parasite and stimulate some resistance response. The most effective way of preventing this happening is to perform all experiments in a sterile environment.

Taking the above factors into consideration it was decided that an *in vitro* approach was the most effective way to study a host/parasite interaction. Such an approach involves

bringing host and parasite tissues together in a controlled sterile environment. In this way variation caused by a fluctuating environment (chemical and physical) and any contaminating microorganisms could be minimised. To reduce genetic and physiological variation it was decided to use only clonal plant material from plants of the same age, and to culture the host and parasite. By doing this their nutrition could be precisely controlled and a route for the application of radioactive isotopes was also made available. It was decided to reduce the size of the host and parasite tissues as much as possible. This would economise on space, allowing larger numbers of experiments to be performed in a given volume.

Having decided the most effective approach it was necessary to choose a suitable parasite and a suitable host.

Choice of parasite

ManyAngiosperm parasites (eg. *Santalum, Striga* and *Orabanche*) invade the roots of their hosts. However , it is easier in this situation to culture and interact shoots rather than roots. Of the Angiosperm stem parasites several habitually infect woody plants eg. *Viscum album* (Salle 1978), *Viscum minimum* (Olson and Kuijt 1986) and Arceuthobium (Broshot and Tinnin 1986). As it is more difficult to culture woody plants it was decided to use herbaceous material. Indeed there are some Angiosperm parasites which predominantly infect herbaceous plants. One of these is *Cuscuta,* and before this work was begun methods already existed for the culture of this parasite (see Maheshwari *et al.*.1980) making this genus particularly suitable.

Cuscuta plants have lightly coloured stems (usually red or orange/yellow) with scale-like leaves. When growing apices of the parasite come into contact with a vertical host stem or petiole the growth pattern changes. The broad anticlockwise circumnutation changes to a tight, anticlockwise coiling around the host. The invasion of the host takes place at the tight coiled region. On the inner side of the coils discrete groups of cells grow out towards the host. From these pad like structures or prehaustoria, the multicellular feeding organs or haustoria emerge and penetrate the host. After an initial penetration of about 2mm, superficial cells of the haustorium develop into individual filaments. These filaments or search hyphae make contact with the vascular bundles of the host and establish vascular connections, differentiating into xylem or phloem depending upon which type of cell they contacted initially within the host bundle. Complete functional xylem vessels extend between the host and parasite (Macleod 1961). Search hyphae in direct contact with the host phloem differentiate into a 'multifingered' transfer cell (Dorr 1969). Subsequently, the contents of the host phloem are transferred to the phloem of the parasite when host cells are induced to become 'leaky' and the parasite absorbs

nutrients from the apoplast (Wolswinkel 1977). The time taken to establish vascular connections between the host and parasite varies depending on the host (Nagar *et al* 1984). The time which elapses between initial coiling of the parasite stem and the formation of a vascular connection is usually measured in days rather than hours or weeks.

Cuscuta species have been found to be able to parasitise many different host plants (Gaertner 1950) although the reports on the responses of host plants to invasion by this organism vary greatly.

The response of host plants to *Cuscuta* Invasion

Early reports suggested that the response of host plants to *Cuscuta* attack was minimal. Pierce (1894) claimed that,"when *Cuscuta* invades its hosts it generally robs them so rapidly and completely that they sooner or later succumb without having combatted its attack by the formation of any new structure and even without showing any renewed growth". Later reports described definite responses and sometimes resistance to *Cuscuta* parasitism. Thomson (1925) found that stems of *Pelargonium* plants adjacent to invading haustoria swell due to large increases in the diameter of the cortex and the development of a cork cambium. In thicker stems when hypertrophy was pronounced, the haustoria of the parasite failed to reach the vascular tissues of the host. Dean (1937) claimed that the development of a gall like structure was a common response to parasitism by *Cuscuta.*and showed that many hosts develop a generalised swelling directly under the coiled region of the parasite. Although the formation of these 'galls' was very frequent they did not always occur. Clearer examples of resistance with evidence of death of haustorial cells has been described by Tsivion (1979). Tomato plants responded hypersensitively to invasion by *Cuscuta campestris.* Cells in the outer cortex of the host became disorganised and died and adjacent cells hypertrophed. This death and hypertrophy of cells of the host was accompanied by death of the invading search hyphae of the parasite. This example shows that host plants can under certain circumstances respond to parasitism by *Cuscuta.*

There are over twenty different species of *Cuscuta. Cuscuta campestris* (field dodder) was the species used in the work described in this thesis. It was chosen because it could be obtained in large quantities. Seeds can be stimulated to germinate with a frequency of 60% by immersion in concentrated sulphuric acid for 1hr, followed by neutralisation in a 10% calcium carbonate solution and then thorough washing in several changes of distilled water before sowing. This species is also smaller than the native *Cuscuta europea* and therefore more easily manipulated in the confined space of the *in vitro* environments. *Cuscuta campestris* was shown by Gaertner (1950) to parasitise many different host species. Only one host plant was selected for this study.

The choice of the host plant

It was important to be able to produce large amounts of clonal plant material. *Coleus blumei, Impatiens sultani* and *Pelargonium zonale* plants can all be propagated easily by root cuttings and *Cuscuta campestris* successfully infects all three. It was envisaged that excised cultured stem from one of these plants would act as the host in an *in vitro* approach. However, it was found that *Cuscuta* shoot tips coiled around the long, slender, vertical petioles of *Pelargonium* more readily than around the thicker stems of *Coleus* or *Impatiens* plants. It was therefore decided to use *Pelargonium* petioles as the *in vitro* host. The successful interaction of sterile host and parasite was preceded by a programme of experimental development.

Development of the *in vitro* approach

Wherever possible during the development of this novel *in vitro* approach attempts were made to standardise the interaction between *Cuscuta* and *Pelargonium.* in order to reduce the variation between individual interactions. Also amounts of host and parasite were kept to a minimum thereby simplifying the system further by eliminating parts of the plants which were not necessary for successful infection to take place. In order to achieve successful infection (a parasite forming vascular connections with a host) *in vitro* the effect of each factor considered to influence the infectivity process was investigated. For example the effect of the size of the inocula and the optimum lighting regime on infection were determined. It was necessary to devise procedures to maintain the host and parasite tissues in culture and ways of bringing them together. Finally it was necessary to confirm that the results obtained with the *in vitro* system were similar to the events which take place *in vivo.* This was achieved by a detailed comparison of the structural changes which take place *in vitro* and *in vivo.* It follows that a large part of this thesis is taken up with a description of the development of an *in vitro* approach. There are two other major sections:-

(1)A description of the morphological and structural changes which take place in the host and parasite during the infection of *Pelargonium* plants by *Cuscuta campestris.*

(2)A description of an investigation into the role of selected cell wall degrading enzymes in the invasion of *Pelargonium* plants by *Cuscuta campestris.*

The importance of a record of morphological and structural changes

This section acts as a baseline for the other sections of this thesis. As already mentioned it was important that host/parasite interactions *in vitro* were representative of those *in vivo.* This record establishes the major changes which occur during infection *in vivo.* In addition to this, structural changes which occur in the host and parasite provide important clues as to the underlying biochemical changes which take place during invasion.

The morphological and structural record was particularly important in interpreting the significance of the changes in the activities of certain cell wall degrading enzymes.

Reasons for investigating the role of cell wall degrading enzymes during infection with *Cuscuta*

Cell wall degrading enzymes have been found to be involved when bacteria (Bashan *et al.* 1985, Davis *et al.* 1986), fungi (Bateman 1976) and Angiosperm parasites other than *Cuscuta* (Reddy *et al.* 1980, 1981) infect the tissues of higher plants. These enzymes may have an important role in the infection of host plants by *Cuscuta.* Since this was suggested by Thomson (1925) little work has been perfomed to substantiate it. Nagar *et al.* (1984) were able to show that the activities of certain cell wall degrading enzymes, notably cellulase, polygalacturonase, and pectin esterase, were much higher in parasite cells adjacent to the points of penetration. Cellulase enzymes degrade the cellulose microfibrils which make up a large part of the primary cell wall. Pectin esterase and polygalacturonase are both involved in the degradation of pectic polymers. The former enzyme is responsible for de-esterifying the side groups of residues in polygalacturonic acid. This increases the number of residues available for attack by polygalacturonase which can only act on those which are not esterified. In this thesis changes in the activities of cellulase, pectinesterase and polygalacturonase during the time course of infection of *Pelargonium* plants by *Cuscuta campestris* were studied and attempts made to relate these changes to the physical and morphological changes. No attempt has been made to do this in previous work. However, such an approach is essential to determine if these enzymes have a specific role in parasitism by *Cuscuta.*

Aims and objectives

The overall objective of this work was to understand the mechanisms by which *Cuscuta campestris* invades a host plant and avoids triggering a response in the host.

There were two major aims within this overall objective. Firstly to develop an *in vitro* system to act as an effective tool to study the host/parasite interaction. Secondly in parallel with this, to observe and record morphological, structural and selected molecular changes which occur in the host and parasite. The detailed aims are now summarised:-

(1) To determine the infectivity and the factors which affect the infectivity of parasite inocula.

(2) To devise a culture procedure for host and parasite and an environment within which the interaction could be studied.

(3) To achieve infection inside this *in vitro* environment.

(4) To establish that the *in vitro* system was representative of the *in vivo* interaction by comparing the structural record of events.

(5) To investigate the role of certain cell wall degrading enzymes in the interaction between host and parasite, paying particular attention to their change in activity with time during the different stages of infection.

Chapter 2

Chapter Two MATERIALS AND METHODS

Section 1 - Plant material

1.1 The parasite

Cuscuta campestris (Yunker). common name 'field dodder' is a member of the family Convulvulaceae. Seed was obtained from the Cambridge University School of Botany and from Inge Dorr at Kiel University in West Germany.

1.2 The host

Pelargonium zonale var Kleine lieblinge is a member of the family Geraniaceae. Plants were obtained by vegetative propagation from existing ones grown in the grounds of the Department of Botany in Edinburgh.

Section 2 - The setting up of interactions between host and parasite *in vivo*

2.1 Growth of plant material

2.1.1 Growth and propagation of *P. zonale*

Large numbers of *Pelargonium* plants were propagated by rooting excised 150mm shoot apices in plastic pots containing John Innes potting compost No. 1. Rooted cuttings were potted on and kept in the greenhouse. Diurnal temperature variation ranged between 15 and 25^{o}C. Natural daylight was supplemented when required by 400W mercury vapour bulbs to produce a daylength of 16hr irrespective of the season.

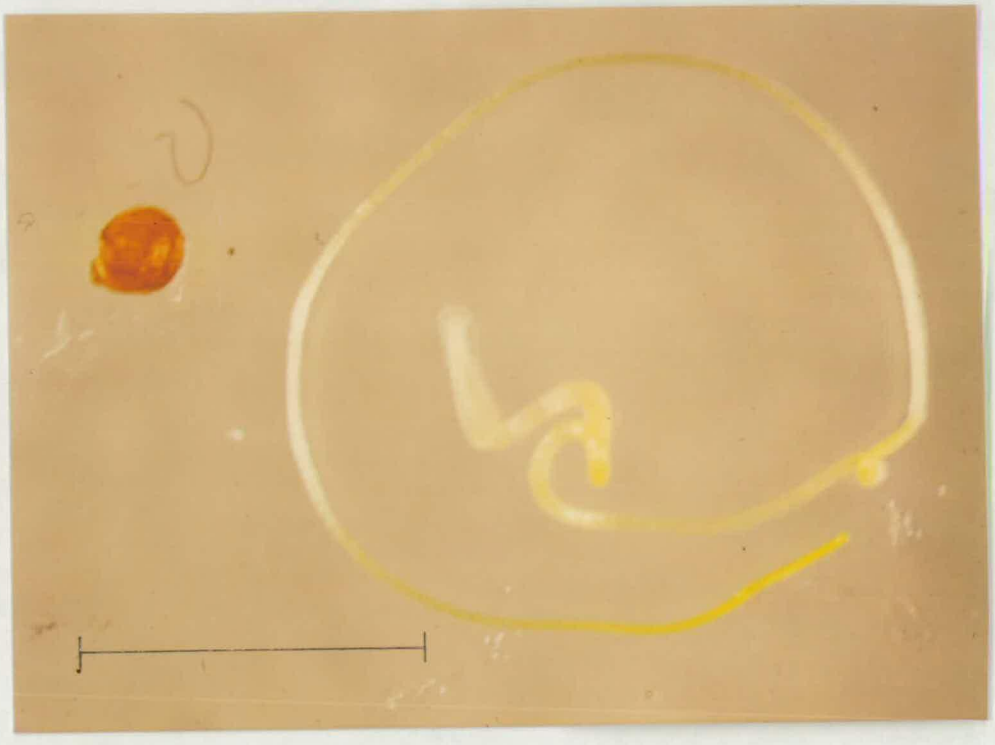
2.1.2 Germination of *Cuscuta campestris* seeds

To remove the inhibition to germination imposed by the seed coat *Cuscuta* seeds were placed in Gooch crucible and immersed in concentrated sulphuric acid for 60min. This was followed by transfer to 10% sodium bicarbonate solution and then two changes of distilled water to remove all traces of the acid. The treated seed was then sown in trays of moist Levingtons compost.

2.1.3 Vegetative propagation of *Cuscuta campestris*

Large amounts of clonal *Cuscuta campestris* vine was propagated from a single seedling (see Fig 2.1.3a). A germinated seedling was placed on the soil at the base of a host plant. The seedling was allowed to locate the host by its spiralling growth pattern and from tight coils around it. The several shoots which subsequently grew from the tight

Fig2.1.3 A newly germinated *Cuscuta campestris* seedling beside the seed coat from which it has emerged Scale = 10mm



coils around it. The several shoots which subsequently grew from the tight coiled region (7-14 days later) were excised and placed within the leaf canopy of other *Pelargonium* plants. As each of these shoots became established further transfers were made.

2.2 Excising and securing *Cuscuta* stem tips onto the Host plant.

Fragile *Cuscuta* stem tips of the required length were carefully excised with a sharp razor. These were attached to *Pelargonium* petioles using small strips of autoclave tape (see Fig 2.2.a). The host and the parasite were held in contact with the length of the shoot tips running parallel with the length of the vertical petiole (see Fig 2.2.b). The apices of the shoot tips were always orientated towards the top (the leaf end). In order to stimulate the shoot tips to coil in the usual anti-clockwise manner particular care was exercised in fastening the apex to the petiole (this is the region most sensitive to stimulation). The concave side of the hooked or curved apical region was wrapped around the cylindrical petiole in an anticlockwise manner (see fig 2.2.c). When fastening was complete the host/parasite interactions were left overnight to allow the parasite to respond to the host. If coiling had not occurred after this period of time it was often necessary to re-orientate the parasite shoot apex because contact with had been lost.

Section 3 - Sterile procedures

3.1 Sterilising heat stable materials

Glassware, metal instruments and chemicals stable during heating were autoclaved at 121^{o}C and 15psi for 20min. Media and Chemicals were autoclaved inside conical flasks sealed with metal foil. Other items were wrapped in two layers of metal foil and sealed with autoclave tape before autoclaving.

3.2 Sterilising heat labile materials

The growth substance indole acetic acid (IAA) was used as a consituent in the growth media in several experiments. Solutions (100mg.ml^{-1}) of this heat labile material were filter sterilised using heat sterilised Swinnex filter units with 0.2μm Millipore filter. Sterile disposable syringes were used to push the solutions through the filter unit.

3.3 Sterilisation of living plant material

3.3.1 Leaves, Stems and Petioles

With the exception of *Cuscuta* seed all plant tissues were sterilised in appropriate concentrations of solutions of sodium hypochlorite plus 0.05% Tepol (detergent). *Cuscuta*

Fig. 2.2a. A diagram showing the manner in which *Cuscuta campestris* shoot tips are inoculated onto *Pelargonium zonale* petioles *in vivo*

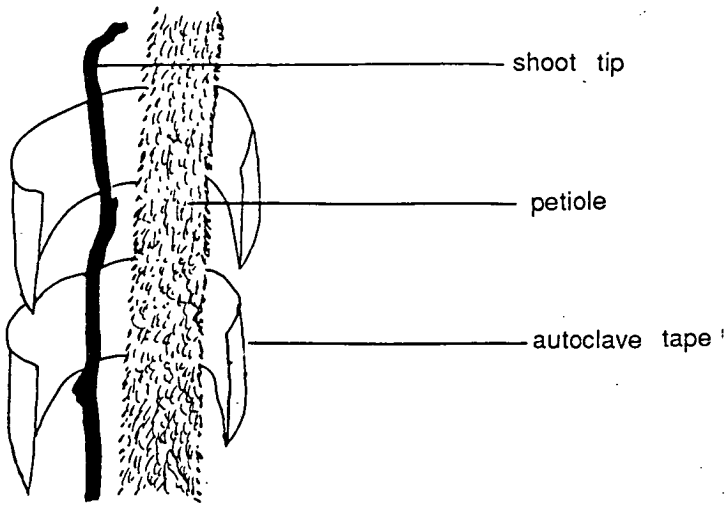


Fig. 2.2b. A diagram showing the close contact between *Cuscuta* shoot tips and *Pelargonium* petioles following inoculation

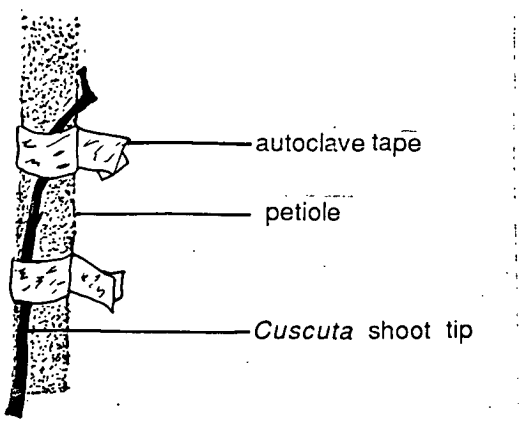
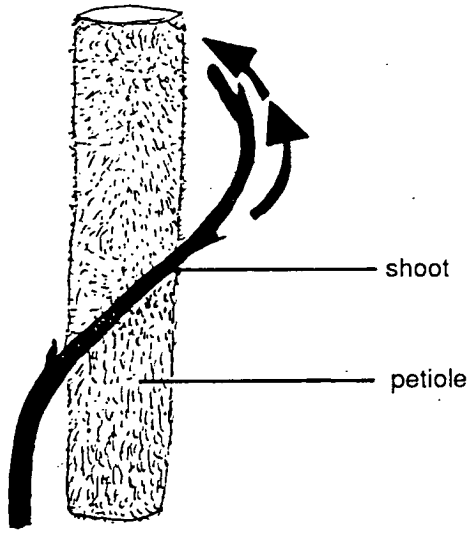


Fig. 2.2c. A diagram showing how the concave side of the hooked or curved apical region of *Cuscuta* shoot tips is wrapped around *Pelargonium* petioles.



shoot tips were washed for 8mins in 5% sodium hypochlorite solution. *Pelargonium* petioles were sterilised for 30min in 10% hypochlorite. The sterilant was washed off with 3 changes of sterile distilled water.

The cut surfaces of excised plant tissues were sealed with wax prior to sterilisation to prevent harmful infiltration of hypochlorite. All subsequent steps were performed inside a Laminarflow cabinet. The sealed plant tissues were then transferred through the sterilising and washing solutions inside glass cylinders (12cm by 2.5cm) as shown in Fig 3.3a. The cylinders were first wrapped in metal foil and autoclaved. When required the metal foil was removed from one end and plant tissues were fed into the cylinder by hand. The open end was then covered with a single layer of muslin held in place with a rubber band. The cylinder was then allowed to slide out of its metal casing (therefore avoiding contact with fingers) into 250ml of hypochlorite of the appropriate concentration in 250ml wide necked conical flask. After gentle shaking to remove any air bubbles the neck of the conical flask was sealed with a sterile metal foil lid. The cylinders were left inside the hypochlorite for a predetermined time and then transferred through a series of 3, 250ml flasks containing 250ml of sterile distilled water (see Reinert and Yeoman 1982). Transfers were made with sterile forceps. The cylinders were left in each flask of water for 5, 10 and 15 min. respectively. When washing was complete the sterile plant tissues were removed from the cylinders and stored in sterile Petri dishes inside the flow cabinets ready for use.

3.3.2 Sterilising *Cuscuta campestris* seed

Cuscuta campestris seed was germinated *in vitro* on agar. The treatment which broke dormancy (see section 2.1.2) was adapted to double as a procedure for sterilising the seed. Approximately 20ml of concentrated sulphuric acid was poured into a sterile 100ml beaker in a flow cabinet. A heat sterilised Gooch crucible containing 100 *Cuscuta* seed was then lowered into the acid. The beaker was covered with a sterile piece of metal foil (see Fig 3.3.2a). After lhr the crucible was removed with forceps to a sterilised metal cage. The crucible was then transferred inside the cage through a series of sterilised solutions. Firstly 10% sodium bicarbonate solution, then two changes of distilled water. Care was taken to keep the lid of the crucible in place to prevent the seeds escaping out of the top. After the second wash the seeds were transferred to a sterile Petri dish. The seeds were then transferred singly with fine forceps to Petri dishes containing 20ml of 1% aqueous agar. Four seeds were transferred to each dish. The dishes were sealed with parafilm and stored in dim fluorescent light 10-15 μmoles $m^{-2}s^{-1}$ at 23°C.

Fig. 3.3a. A diagram showing how petioles or shoot tips are held inside glass cylinders while being sterilised and transferred from solution to solution.

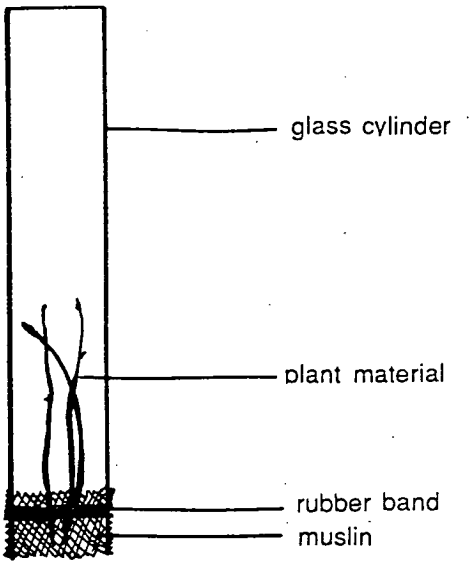
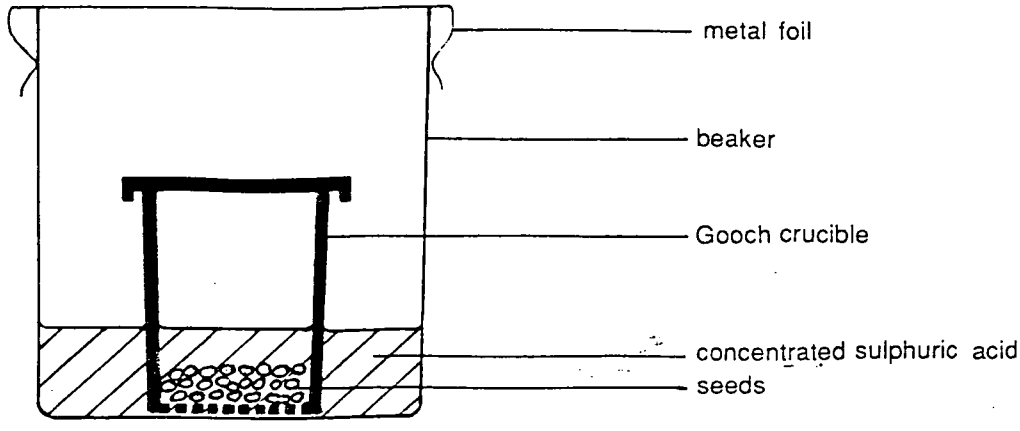


Fig. 3.3.2a. A diagram showing a Gooch crucible containing *Cuscuta campestris* seeds immersed in concentrated sulphuric acid inside a sterile sealed beaker.



3.4 Maintaining sterility during experiments

All experiments were carried out inside Laminar flow cabinets. The cabinets were regularly wiped down with cotton wool soaked in 100% ethanol.

3.5 Resterilisation during experiments

In between manipulations forceps and scalpels were kept in glass jars with their working ends immersed in 100% ethanol. When the instruments were required they were resterilised by passing through a bunsen flame which ignited the ethanol on the surface. When the ethanol was completely burned off the instruments were held in a stream of sterile air for a few seconds to cool before being used.

3.6 A method for *Cuscuta* shoot tip culture

The method used was based on that of Maheshwari (1980). *Cuscuta* shoot tips were cultured with their cut ends bathed in liquid culture medium in Durham tubes (7mm by 35mm). The tubes were supported in 1% agar inside specimen tubes (see Fig 3.6a).

Assembly of the culture apparatus

Approximately 2.5ml of 1% molten aqueous agar was poured into each specimen tube (100 x 25mm). A metal foil lid was placed on the tubes before they were autoclaved at 121^{o}C for 20 min. All the succeeding steps were performed inside a Laminarflow cabinet. Sterile empty Durham tubes were pushed into the agar at the base of the specimen tubes with long forceps. Approximately 1.5 ml of liquid medium was placed in each tube with sterile long nosed Pasteur pipettes. The tubes were then sealed and stored ready for inoculation with a single shoot tip.

Growth medium

The growth medium was a modified Modified Whites medium (see Table 3.6) similar to that used by Maheshwari (1980). Before inoculation into the growth medium the shoot tips were sterilised.

Sterilisation of the shoot tips

Unbranched 40mm *Cuscuta* shoot tips were excised with a sharp razor blade from clonal greenhouse vines parasitising *Pelargonium* plants. The cut ends were immediately sealed with molten wax. All subsequent manipulations were performed inside the Laminarflow cabinets. Groups of 8 shoot tips were sterilised by the method described in section 3.3.1, treating with 5% of saturated aqueous hypochlorite for 8 mins.

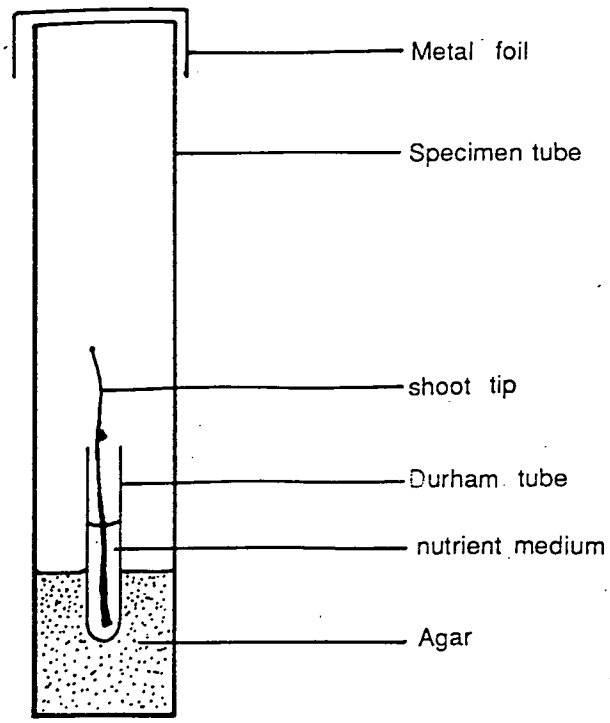
When washing was complete the fragile shoot tips were transferred to sterile Petri

Table 3.6. The medium used to culture *Cuscuta* shoot ships, a modified Whites medium.

Substance	Quantity $mg.l^{-1}$
Mg. $SO_4.7H_2O$	720
Ca $(NO_3)_2.4H_2O$	208
Na_2 SO_4	200
Na $H_2PO_4.2H_2O$	200
KNO_3	80
KCl	65
Fe EDTA	4.59
Zn $SO_4.7H_2O$	3.00
H_3 BO_3	1.50
Cu $SO_4.5H_2O$	0.001
Glycine	3.00
Thiamine HCl	0.10
Nicotinic acid	0.50
Pyridoxin HCl	0.10
KI	0.75
Mo 0_3	0.0001
Sucrose	40,000

To this GA_3 was added to a final concentration of 1×10^{-4}M.

Fig. 3.6a. A diagram showing how excised *Cuscuta campestris* shoot tips are cultured in liquid culture medium inside tall specimen tubes.



dishes. Each tip was trimmed to 30mm with a sharp scalpel before being carefully inoculated with long forceps into the liquid growth medium. Sealed specimen tubes were incubated at 23°C on a shelf under fluorescent lighting (10-15 μ moles $m^{-2}s^{-1}$)

3.7 The culture of *Pelargonium* petioles in vertically held Petri dishes

Parkinson and Yeoman (1982) used an *in vitro* technique for culturing excised internodes of several Solanaceous plants. This method described here to culture *Pelargonium* petioles was an adaptation of their technique.

Excised sterilised petioles were sandwiched between two layers of agar growth medium in a vertically held Petri dish (see Fig 3.7).

Petri dish preparation

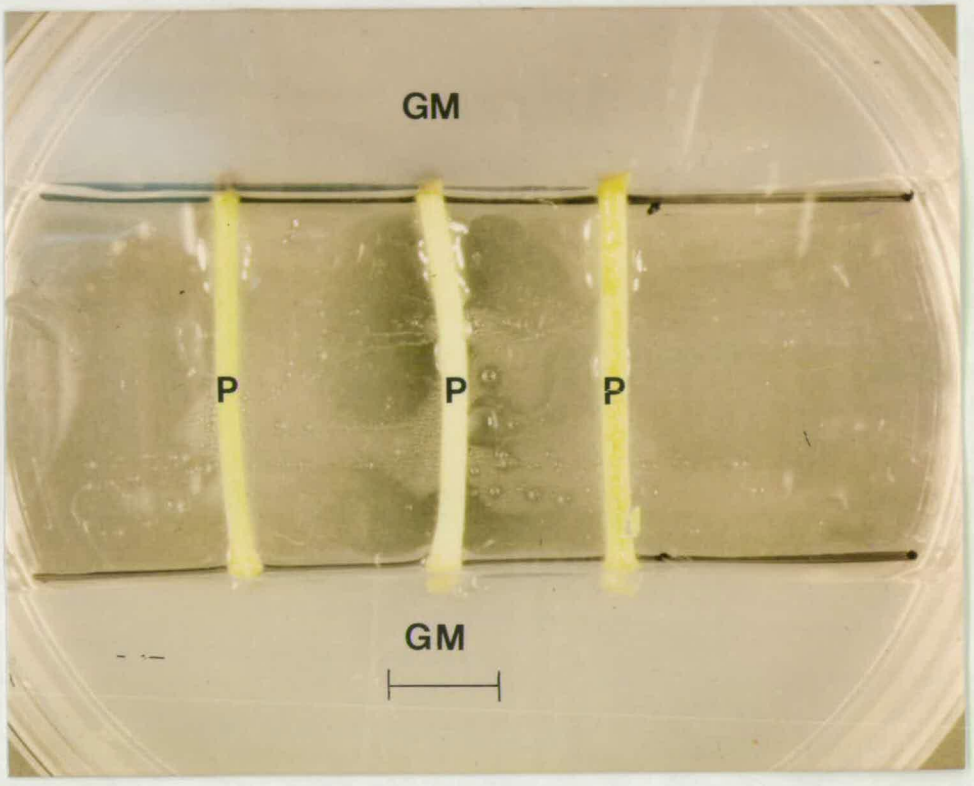
A sterilised 12mm metal spacer was inserted in the middle of a sterile Petri dish dividing the dish into two. The two sides were designated, top and bottom. Approximately 10ml of molten M and S growth medium (plus 2% sucrose 1% agar and 0.2mg l^{-1} kinetin) was poured into the bottom section. An equal volume of the same medium plus 0.2 mg l^{-1} IAA was added to the top section. When the agar had set the metal spacer was removed. The space between the agar was then increased to 32mm by removing a further 10mm from each side with a sterile scalpel.

Petiole preparation

Petioles from newly emerged leaves were chosen for culture. A 50mm length of petioles was excised with a sharp razor blade. The stem end was cut at right angles and the leaf end obliquely so that polarity could be identified throughout the experiment. Both ends were sealed with wax. The excised petioles were sterilised in groups of 10 with 10% sodium hypochlorite for 30 mins in the way described in section 3.3. Following washing the petioles were transferred to sterile Petri dishes. Each petiole was trimmed to 36mm with a sharp scalpel and then inserted in spaces between the two sections of medium in the Petri dishes. The ends of the petioles protruded into the growth medium. The leaf ends of the petioles were orientated towards the top section of the dishes to preserve polarity. Three petioles were cultured in each dish. The Petri dishes were sealed with a parafilm and then taped flat against the wall with the top section at the top in a culture room continuously lit with a fluorescent light at an intensity of 10-15 μmoles $m^{-2}s^{-1}$ and at a temperature of 25°C.

Section 4 - Microscopical examination of plant materal

Fig 3.7 The split plate system for culture of *Pelargonium* petioles. (P) = petioles (GM) = Growth medium. Scale = 10mm ▼



4.1 Preparation of specimens

4.1.1 Preparation and sectioning for Light (LM) and Transmission Electron Microscopy (TEM)

All the plant material for LM and TEM was processed in the same way unless otherwise stated. The parts of the plants selected for microscopic examination were dissected out from larger parts which were submerged in the primary fixative. The primary fixative used was one of two types: (a) A 0.05M phoshate buffer at pH 7.2 containing 4% glutaraldehyde. The phosphate buffer was made by mixing 36ml of 0.2M Na_2HPO_4 and l4ml of 0.2M NaH_2PO_4 which was then diluted to 0.05M with the other constituents of the fixative. (b) Karnovskys fixative, which consisted of a 0.1M cacodylate buffer pH 7.2 containing 2.5% glutaraldehyde and 2% paraformaldehyde. Sodium cacodylate buffer was a mixture of 50ml 0.1M sodium cacodylate trihydrate $Na(CH_3)\ As0_2\ 3H_20$ and 4.15ml 0.1M HCl diluted to 100ml with distilled water. Specimen tissues were left submerged in fixative overnight at 4°C. The primary fixative was then washed out by three l/2hr washes in buffer prior to a 2hr secondary fix in 2% aqueous Osmium tetroxide ($0s0_4$). Traces of the secondary fix were removed by three 1/2hr washes in distilled water. This was followed by deyhdration through an ethanol series (2hr in 20, 40, 60 and 80%, 4hr in 96%, 2 (l2hr) in absolute and overnight in dry absolute). Ethanol was kept dry by storage with a molecular sieve (BDH Chemicals Ltd. Poole, UK). Specimens were embedded in resin. Specimens were transferred to resin via an ethanol/propylene oxide and a propylene oxide/resin series (2hr each in 3:l, 2:l, l:l, l:2, l:3 ethanol/propylene oxide and 2hr in pure propylene oxide followed by 6hr in 3:l and 2:l, l2hr in l:l, l:2, l:3 propylene oxide/resin and l2hr in complete resin). After a further change of pure resin the specimens were transferred to fresh resin in Beem embedding capsules and placed in an oven at 60°C for 48hr. The specimens were embedded in one of two resin mixtures:

(a) Araldite CY212.....50.0 g
Dodecyl succinic anhydride (DDSA)...50.0 g
Benzyl dimethylamine (BDMA).....2.5 g

(b)
Epikote 812 substitue.....25ml
Dodecyl succinic anhydride (DDSA).....2.5ml
Dibutyl phthalate (DBP).....2.5ml
Benzyle dimethylamine (BDMA).....1.25ml

Sections were cut on a Reichert "Ultracut" ultatome using 45° glass knives made on a LKB knife maker (type 7801A). Sections for LM were cut 0.5, 1.0 and 1.5 μm thick and sections for TEM were 50-100 nm thick appearing silver and gold when viewed on the microtome. Sections for light microscopy were picked up with a platinum loop from the surface of the filtered distilled water reservoir behind the knife edge. They were then placed on drops of distilled water on new glass slides (Berliner Glas K.G., Berlin D.D.R.) The slides were transferred to a hot plate at 60°C and the sections allowed to expand and dry down onto the glass. Alternatively sections were transferred to stain solutions for the requisite period before being dried down onto the slides. Ultrathin (silver/gold sections for EM floating on the surface of the water were expanded by holding a swab soaked in chloroform 1-2cm above the surface. They were then lifted off the surface of the water onto copper or gold grids of varying grid size and bar thickness (Agar Aids, Stanstead, U.K.) and stored in a dessicator until examined.

4.1.2 The preparation of specimens for Scanning Electron Microscopy (SEM)

The initial stages of the preparation of specimens for SEM were identical to those for preparation for Light and Transmission Electron Microscopy. Primary and secondary fixations were performed as described in section 4.1.1. Following fixation specimens were dehydrated by diffusion dehydration. Each specimen was placed in 5ml of 20% acetone in a glass vial on a shelf inside a desiccator. The base of the desiccator was filled to a depth of 1.25cm with calcium sulphate solid and then covered with the same volume of 100% acetone. A watch glass of anydrous calcium chloride was placed on the desiccator shelf at the side of the sample. (see apparatus in Fig 4.1.2a) The desiccator was then sealed and the pressure inside reduced with a vacuum pump until the acetone was seen to boil. The specimen was left under vacuum overnight and then moved to another dessicator at atmospheric pressure.

In order to remove the acetone the specimens were then critical point dried in a Poloron bomb type critical point drier. The specimens were infiltrated with CO_2 for 1 hr at 32°C and a pressure between 1200 and 1600 psi. Following this specimens were attached to SEM stubs with silver dag. The stubs containing the specimens were then coated with gold using an Emscope Splutter Coating Unit. Each specimen was bombarded with gold for 3-4 min. The specimens were now ready for observation in the SEM. In between observation they were stored in a dessicator.

4.2 The equipment used in the different types of microscopy

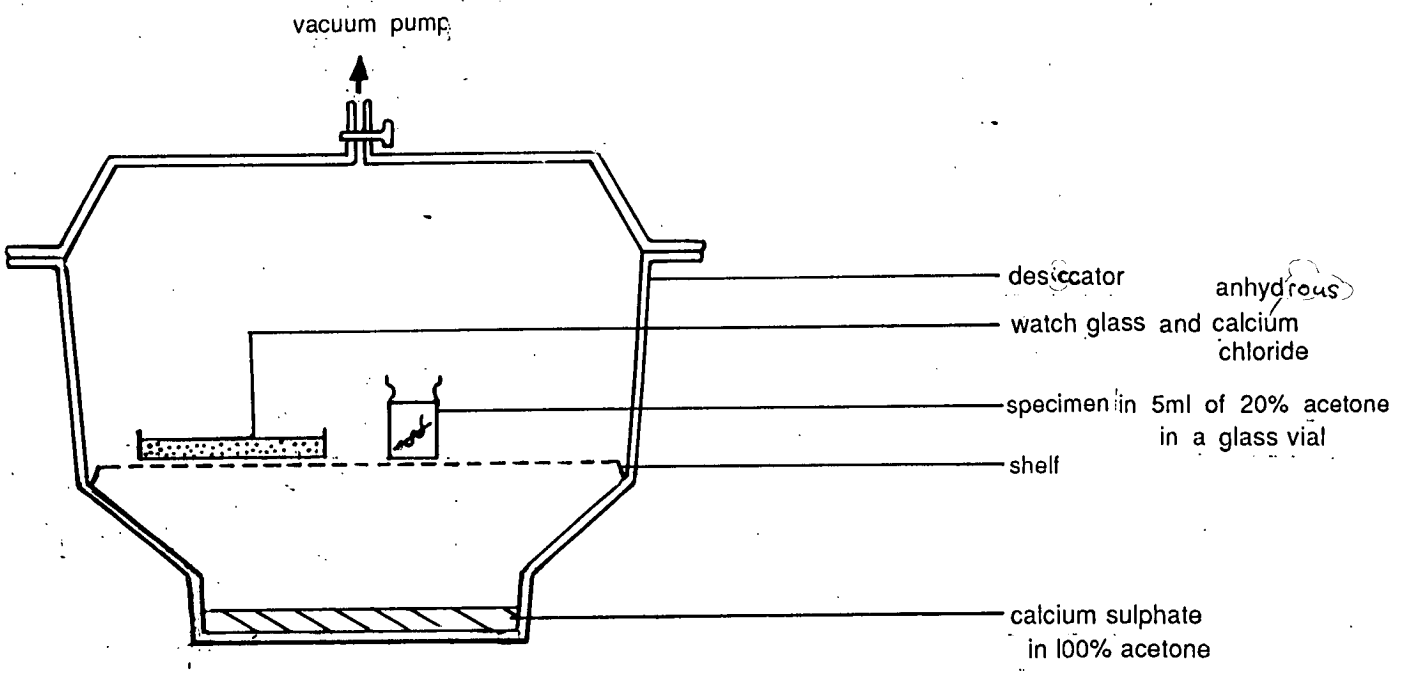


Fig. 4.I.2a. A diagram showing the apparatus used in the rapid dehydration of plant material during preparations for S.E.M.

4.2.1 Light microscopy

Light microscopy was performed on a Reichert Plyvar photomicroscope. Stained slides were photographed on Kodak technical Pan 2415 (development 6min in 10% v/v Kodak HC110 in water at 20°C). Micrographs were printed on Ilfospeed multigrade paper.

4.2.2 Transmission Electron Microscopy

Silver/gold sections were examined on a Joel 100S electron microscope at an accelerating voltage of 80 kV, or 60 kV if specimen contrast was low. Specimens were photographed on Ilford P.Q. and developed with Universal developer. The developer was made up as a 10% v/v solution in water containing a few drops of Ilford Ilfotol wetting agent and the film was developed for a 4 minutes at 20°C.

4.2.3 Scanning Electron Microscopy

Coated specimens were viewed in a Cambridge Instruments S150 or S250 microscoped. The specimens observed at an accelerating voltage of 1.0 to 2.5 kV and condenser lens (Cl, C2) currents between 0.75 and 1.25 amps.

Areas to be recorded were selected and photographed as rapidly as possible. The duration of the raster scan was set at 15, 30 or 60 seconds. Negatives were made on Kodak Plus X Pan professional film which was developed in a 12.5% v/v solution of Kodak HC110 developer for 5 min at 20°C.

4.3 Staining procedures

4.3.1 Toluidine Blue 0 staining of resin embedded sections cut for light microscopy

Toluidine blue was used as it is a general purpose stain widely used for epoxy sections as it stains both cell walls and cytoplasmic constituents (Trump et al 1961). After the sections were completely dried down onto the glass slides they were stained for 2-5 min in a filtered 1% w/v aqueous solution of Toluidine Blue (B.D.H. Poole, U.K.) made up in a 1% w/v sodium borate solution. The slides were then rinsed thoroughly with distilled water followed by rinsing in 70% ethanol (in order to produce metachromatic staining). Stained slides were then dried on a hotplate at 60°C before being mounted in resin.

4.3.2 The staining of thin sections with Uranyl acetate / Lead citrate

The staining with Uranyl acetate was performed in the dark inside black glass Petri dishes. This substance forms a precipitate in the presence of light.

Rectangles of washed dental wax were placed inside the black Petri dish. Discrete drops of filtered (with a Swinnex filter unit containing a 0.2µm Millipore membrane) aqueous Uranyl acetate were put quickly onto the top of the wax and the lid replaced. Grids containing thin sections were placed face down onto the drops and left for 1hr. The grids were then removed with forceps. While still held the grids were quickly washed by running drops of distilled water down the forceps towards the ends. After careful drying in air the grids were ready for staining with Lead citrate.

Lead citrate forms a precipitate in the presence of air. Staining was therefore performed inside a sealed Petri dish containing filter paper soaked in 2M sodium hydroxide (NaOH) and 30-40 NaOH pellets. Washed dental wax was placed on top of the filter paper adjacent to the pellets. Discrete drops of filtered (filtering as for Uranyl acetate) Lead citrate were then placed on the surface of the wax before quickly resealing the Petri dish. The grids ready for staining were placed face down on the drops of Lead citrate and the dishes then quickly resealed. The staining was allowed to proceed for 10 min before the grids were removed with forceps and carefully washed with 2M NaOH and then distilled water while still held. When the grids were dry they were stored in a specially designed case, (Agar Aids).

The lead citrate solution was made by adding 1.33g of lead nitrate and 1.76g of trisodium citrate to 30ml of distilled water. After shaking vigorously for 1min the solution was stirred gently for 30min. Then 8ml of 1N NaOH was added and the mixture made up to 50ml with distilled water.

Section 5 - Protein Extraction, Purification and Estimation

5.1 Extraction Procedures

5.1.1 Extraction of Protein by a grinding method

In a number of experiments the following method of extraction was used. Unit weight of plant material was ground in a mortar and pestle with twice the weight of ice cold extraction buffer (1.OM NaCl in 0.02M Tris/HCl pH 8.1 + 3mM EDTA disodium salt. Tris/HCl buffer was made by mixing 50ml of 0.2M Trizma base with 26.2ml O.2M HCl and diluting ten fold with distilled water. Acetate buffer was made by mixing 0.5M Sodium acetate solution with 0.5M Acetic acid in proportions which gave the correct pH and diluting ten fold with distilled water) and 10% acid washed sand. After thorough grinding the homogenate was left to stand on ice for 15min before further grinding for one min to redisperse. The homogenate was expressed through four layers of muslin into an ice cold plastic beaker before being transferred to polycarbonate centrifuge tubes and spun at

13000rpm (20 000g) for 20min at 4°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge using a Sorval SS- 34 head). When spinning was complete the supernatant (containing the protein) was removed from the pellet and stored on ice in the cold room.

5.1.2 Extraction of Protein by a freeze/thaw method

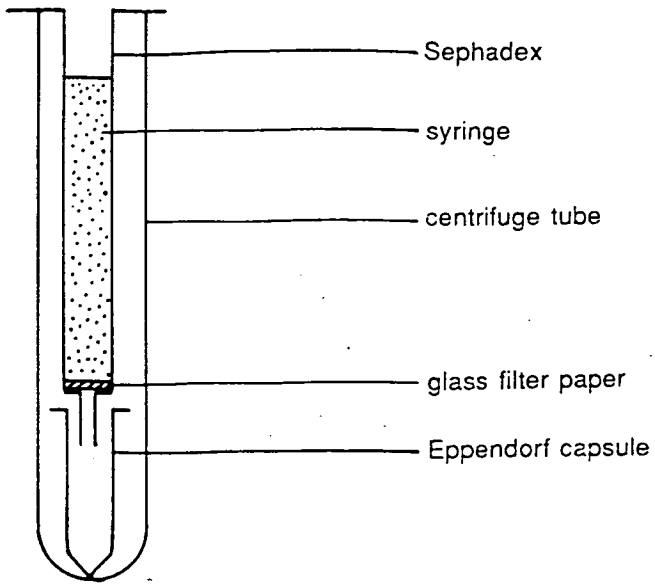
When the amounts of plant material were 1g or less an alternative extraction procedure was used. Samples were weighed using a Sartorius digital balance before being chopped into small pieces (approximately 2 by 1 by 1mm) and added to twice the weight (assuming the $1cm^3$ of plant tissue = 1g) of Tris/HCl or Acetate extraction buffer (for details see section 5.1.1) in Eppendorf capsules. The Eppendorf capsules were transferred to a -20°C freezer for 30min and then returned to the cold room for 15min. This procedure was repeated twice before storing the capsules overnight on ice in the cold room. After centrifugation in an Eppendorf 5412 at 12 000 rpm for 15min the supernatants were transferred to empty capsules and stored on ice in the cold room.

5.2 Purification and concentration of Protein extracts

5.2.1 Rapid removal of salt from crude extracts by a spun Sephadex column method

This procedure used centrifugal force to accelerate gel filtration of small volume (100-500ul) of crude protein extracts. Small columns of Sephadex G50- 150 (SIGMA, St Louis USA) were made up inside disposable syringes. The size of the syringe chosen depended on the size of the protein sample to be filtered. Samples were filtered in syringes which were 10 times their volume. Sephadex was firstly autoclaved with 20 times their own volume of distilled water before being pipetted into syringes which had their bottoms plugged with a single circle of glass filter paper (see Fig 5.2la). The autoclaved Sephadex was added until the sedimenting beads reached the top graduated mark on the syringe. The column was then equilbrated with the buffer of the type the protein sample was required to be in at the end of filtration. Equilbration was achieved by washing the Sephadex with four to five column volume of ice cold buffer. The syringe was then inserted into a polycarbonate centrifuge tube and spun at 1500g for 4min at 4°C. Eluate resulting from the spin was removed and an Eppendorf capsule (with the lid removed) placed at the base of the syringe (see Fig 5.2.1a). A sample of buffer identical in volume to the crude protein sample to be filtered was carefully pipetted onto the top of the Sephadex. The column was then respun at exactly the same speed and for exactly the same time as before. After removing the eluate the Eppendorf was replaced and the crude

Fig. 5.2.Ia. A diagram showing the apparatus used to remove salts from crude protein extracts.



protein sample carefully added to the top of the Sephadex. The column was spun again for the same speed and for the same time. This final spin yielded purified protein sample which was transferred to a fresh Eppendorf capsule and stored on ice in the cold room.

5.2.2 Removal of salt from crude extracts by dialysis

This method was used when the spun column method was found to be not suitable for purifying *Pelargonium* extracts due to low yields of protein in the eluate. The dialysis tubing was first boiled in 10mM EDTA (the disodium salt for 30mins to removed traces of enzyme damaging heavy metals). Once washed with distilled water and drained the tubing was sealed at one end by knotting. The ice cold crude extract was carefully poured into the tubing and sealed in by a further knot. Care was taken to ensure that the resulting sac was turgid and contained no air bubbles. The dialysis sac was then totally immersed in ice cold buffer 500 times its own volume but containing no salt, EDTA or DTT. The buffer was kept stirred constantly in the cold room overnight. Two changes of buffer were made after 2 and 4 hrs. The desalted protein extract was removed from the dialysis sac by making a careful incision at one end with a scalpel. The extract was then stored on ice in the cold room.

5.2.3 Concentration of protein extracts by freeze drying

In some instances this method made it possible to detect enzyme activities undetectable in standard protein extracts.

Desalted protein extracts were divided into 1ml aliquots and pipetted into Eppendorf capsules on ice. Opened capsules were placed in a Speedvac concentrator (Savant) and rotated under vacuum at -10^{o}C for one hour. Concentrated samples were then removed and stored on ice in the cold room.

5.3 Estimating the quantity of protein in crude, concentrated and purified protein extracts.

5.3.1 The estimation of protein content by a Bearden modified Bradford method

This method was used in all the estimations of protein content. It was taken from J.C. Bearden (1978).

The protein samples which were assayed were between 20 and 5µl in volume. These samples were first made up to 1.5ml with 0.05M acetate buffer (pH5). The enlarged samples were added to an equal volume of assay reagent (Coomassie Brilliant Blue G-250 Raymond A. Lamb, (London) dissolved in 85% w/v phosphoric acid at a concentration of

1mg.ml^{-1}). This was diluted five fold with distilled water, filtered to remove undissolved solids and then stored at room temperature. The reagent had shelf life of one month at room temperature). Once the protein solution and reagent had been thoroughly mixed with a Whirlimixer (Fisons) the samples were transferred to glass or plastic spectrophotometer cuvettes (the protein-dye complex binds to quartz).

The absorbance was measured with a Pye Unicam SP8-100 Ultraviolet double beam spectrophotometer. Firstly the reading of the protein sample versus the blank (dye reagent plus buffer) was set to zero with the baseline control at 465nm. The wavelength was then changed to 595nm and the absorbance read directly. In order to calculate absolute values of protein standard curves were drawn. Aliquots of 10, 20, 30, 40, 50 and 60µg of protein were incorporated into reaction mixtures and their absorbances at 595nm recorded. The standard protein was Bovine Serum Albumen at a concentration of 1mg.ml^{-1} (SIGMA, St Louis, USA) dissolved in 0.05M sodium acetate buffer pH5. The absorbance value of the standards were plotted against their concentrations. Quantities of protein present in the protein extracts were estimated by reference to this standard curve.

Section 6 - Enzyme Assays

6.1 A Viscometric assay for measuring cellulase (CE) activity

The basis of this assay is that when solutions of the enzyme CE are added to solutions of Carboxy Methyl Cellulose (C.M.C.) the viscosity of the mixture decreases with time. It is possible to relate this change in viscosity with the activity of the enzyme. When the temperature, pH, concentration of substrate and relative concentrations of enzyme extracts and substrate are standardised it is possible to calculate the number of units of cellulase per ml of extract (see Almin and Eriksson 1967a,b and Hulme 1971). The tables for calculating the number of cellulase units per ml of extract used here (see appendix 2.6.1) were calculated by L. Lewis (personal communication) and based on the above papers.

The substrate was made by gradually adding with stirring 5g of C.M.C. 7H3SF powder (Hercules powder Company, London, W1H 8AL) to 500 ml of 0.02M sodium phosphate buffer pH 6.1. When most of the C.M.C. had dissolved the container was sealed with aluminium foil and autoclaved for 15min at 125^{o}C and 15psi. The stirring was then continued for a further 15min. To prevent bacterial contamination 0.02% sodium azide was added to the viscous C.M.C. solution before separating it into 10ml aliquots which were stored in sterile screw topped plastic tubes at 4^{o}C.

It was possible to assay both crude and purified protein extracts with this

procedure. Assays were usually performed using crude extracts. In all the CE assays 0,02M Tris/HCl extraction buffer (pH 8.1) was used. The extracts were obtained by either of the methods described in sections 5.1.1 or 5.1.2. The CE activity in the crude extracts was estimated by measuring the change in viscosity of a mixture a small sample of extract (usually 0.1ml) with twice the volume of substrate (C.M.C.) The change in viscosity with time was measured using calibrated 0.1ml blowout pipettes. Each pipette was calibrated with water. Water was drawn into the pipettes by suction and the meniscus held a. the zero mark by keeping the top sealed. Then the meniscus was allowed to fall simultaneously with the starting of a stop clock. After exactly 2 sec the fall was halted by resealing the top of the pipette. The point which the meniscus had reached was marked with an indelible marker. This process was repeated twice as a check before the pipette was completely drained. The viscosity of the reaction mixtures were estimated by measuring the time it took for their maniscus to fall between the zero mark and the calibration point.

Each 0.1ml protein sample to be assayed was vigorously vortexed with 0.2ml C.M.C. in a $5cm^3$ glass tube for 15s before being placed in a 23^oC water bath in a constant temperature room. After a further 45s the mixture was drawn up into a calibrated pipette beyond the zero mark and then allowed to fall. As the meniscus passed zero a stop clock was started and stopped when it reached the calibration mark. The time taken to fall between the two points were recorded. The assay mixture was returned to the glass tube for further incubation before the pipette was rinsed out with distilled water. The time taken to fall between the two calibration points was recorded in duplicate every 30 min until the viscosity decreased to half its original value. If this was not achieved with 5hr the tube containing the reaction mixture was sealed (to prevent drying out) with parafilm and left overnight before further readings were taken.

Control reaction mixtures consisted of 0.1ml autoclaved protein extract and 0.2ml phosphate buffered C.M.C. and extraction buffer plus 0.2ml phosphate buffered C.M.C. When readings were completed flow times recorded in the experiment were referred to the tables. The tables were calculated so that the time taken for the meniscus of a reaction mixture to fall between two points that it took the meniscus of water 2s to pass through could be converted into a unit of viscosity. For each flow time the table gave a corresponding viscosity value. The enzyme activity per ml of extract could then be worked out as follows:-

$$\text{Number of units of cellulase activity per ml of extract} = \frac{\text{table value for the flow time after x hr} - \text{table value for the flow time at the beginning of the experiment}}{\text{time since the start of the experiment (in hours)}}$$

Protein assays were performed on the residual extract from each sample. This allowed enzyme activity to be calculated on a per mg protein basis.

6.2 A viscometric assay for measuring Polygalacturonase (PGURASE) activity

This method was very similar to the previous method described in section 6.1. The differences are as follows:-

(1) The substrate is a 1% solution of polygalacturonic acid (PGURA) IN 0.05M sodium acetate buffer pH 5.0. This was prepared by stirring 25g of PGURA (sodium salt grade 2, Sigma, St Louis) into 250ml of hot distilled water until completely dissolved. Once cooled the PGURA was dialysed at room temperature against four, 2l changes of 0.05M sodium acetate buffer pH 5.0 containing 0.02% sodium azide. Changes were made at twelve hourly intervals. The substrate was then divided into 10ml aliquots and stored in screw topped plastic tubes at $4^{o}C$. The buffers were made up as described in section 6.1.

(2) Sodium acetate extraction buffer (see section 5.1) was used in all cases to extract the protein for the PGURASE assays.

(3) The amount of protein extract and substrate in the reaction mixture were 50µl and 350µl respectively.

(4) The calibration marks on the pipettes were made so that they represented the distance the meniscus of a column of water dropped in 3s.

(5) The table giving the corresponding viscosity values for reaction mixtures with a particular flow time were compiled by Roy Sexton (personal communication see Appendix 2.6.2). The equation used in combination with these tables is as follows:-

$$\text{Enzyme activity} = \frac{\text{Table value for flow time at time zero t1} - \text{Table value for flow time at time t2}}{t2 - t1 \text{ (in hr)}}$$

6.3 A viscometric method for detecting pectin esterase (PE) activity in protein extracts

Pectin esterase is an enzyme which acts on esterified galactopyrosiduronic residues of pectic polymers liberating methanol and generating carboxyl groups. The action of the enzyme leads to a fall in the pH and an increase in viscosity in the presence of polyvalent cations such as calcium. The latter of these characteristics was exploited in this assay.

Crude protein extracts were made from tissues of *Cuscuta and Pelargonium* by either of the methods described in sections 5.1.1 and 5.1.2. In all cases extractions were performed using Tris/HCl extraction buffer (see section 5.1). The presence of PE was determined in one of 2 ways:

(a) The crude extract samples were divided into 2 portions. A 50µl aliquot of the first portion was vortexed with 350µl of 1% PGURA in 0.05M sodium acetate buffer pH 5.0 in a glass 5cm^3 boiling tube. After a further 45s the viscosity of the mixture was determined in the same way as in section 6.2. This was counted as the viscosity at time zero.

The second portion of the protein extract was boiled for 15 min, cooled, then treated in the same way as the first portion. The above was repeated twice with other extracts. In addition three 50µl aliquots of extraction buffer were treated in the same way to act as further controls.

All the tubes were incubated at 23^{0}C in a water bath. After 2, 4 and 24hr the viscosity of each of the reaction mixtures was remeasured. An increase in the viscosity in the tubes with unboiled protein and not in the other reaction mixtures indicated the presence of PE activity.

(b) Each crude protein sample to be tested for PE activity was first purified by spun column method (see section 5.2.1). From each purified sample 2, 50µl aliquots were taken and used as constituents in the following reaction mixtures:

Quantities shown are in microlitres (µl)

CONSTITUENTS	Reaction mixtures 1	2	3	4
purified protein in 0.02M Tris/HCl buffer pH 8.0	50	50	-	-
0.98M $CaCl_2$ in 0.02M Tris/HCl buffer pH 8.0	50	-	50	-
0.02M Tris/HCl buffer pH 8.0	-	50	50	100
PGURA in 0.05M sodium acetate buffer pH 5.0	600	600	600	600

Each reaction mixture was set up in a glass $5cm^3$ boiling tube. The contents were vortexed for 15s, then after a further 45s the viscosity of this was determined in the same way as in (a). This was taken as the zero time viscosity. The viscosity was remeasured at 2, 4 and 24hr. The tubes were incubated at 23^oC in a water bath. A large increase in the viscosity occurred in RM1 when PE was present in the reaction mixture often leading to complete solidification. Reaction mixtures containing protein extracts but no calcium do not experience any marked increase in viscosity. As PE generates carboxyl groups the calcium ions link adjacent pectic chains leading to the solidification of the reaction mixtures. when calsium is not present (see RM2) no dramatic change in viscosity occurs. Calcium does increase the viscosity of polygalacturonic acid solutions to a some extent by linking together any unesterified residues. However, without PE which greatly increases the number of these unesterified residues the changes are only very limited.

6.4 A quantitative assay for the measurement of Pectin Esterase (PE) activity

Theory

This method is based on that of Kerstesz (1955). When PE is added to solution of PGURA the action of enzyme liberates methanol and generates carboxy groups. The pH of the mixture decreases with the release of protons. The enzyme activity is directly related to the rate of proton production and the fall in pH. In this assay the activity of the enzyme extract is measured by the amount of 0.02M sodium hydroxide required to maintain the pH of the extract in 300 time its volume of a 1% solution of PGURA at pH 7.5 for 1hr. As it requires 1 mole of hydroxide ions to neutralise 1 mole of released protons it was possible to calculate the number of mg or moles of methoxy groups liberated in 1hr per ml of extract, gram of tissue or per mg protein.

The assay

Crude protein extracts were prepared by either method described in section 5.1. Stock solutions of substrate were prepared by dissolving solid (Polygalacturonic acid grade 2, Sigma) in 0.1M sodium chloride with gentle heat to a final concentration of 1%. The solutions were protected from bacterial contamination by adding sodium azide to a final concentration of 0.02% and storage in the refrigerator.

When required a 15ml sample of stock solution was placed in a round bottomed glass tube (internal diameter 2cm). The pH of the solution was adjusted to 7.5. The tube was then transferred to a beaker of water sitting on top of a hotplate/stirrer. Magnetic fleas were placed in both the tube and beaker. The temperature of the water was kept at 30^{o}C and the solutions in the beaker and tube were stirred constantly. The pH of the PGURA was monitored by inserting a pH electode into the glass tube.

PE activity in individual protein samples was estimated by adding a 50µl aliquot of extract to the stirring substrate. Drops of 0.02M sodium hydroxide were added to the reaction mixture from a graduated burette at a rate required to keep the reaction mixture at pH 7.5 for 15 min. The amount of protein added to the substrate was calculated by performing assays on known volumes of residual extract. These protein assays were performed as described in section 5.3.1. The activity of the enzyme was expressed in ml of 0.02M NaOH required per hour to keep the pH at 7.5 per ml of extract.

To show that the change in the pH was due to the proteins present in the extracts a second aliquot was taken from each sample and assayed. However the aliquot was boiled before it was added to the substrate.

6.5 A reducing sugar assay for measuring polygalacturonase (PGURASE) activity

This method is based on that of Riov (1974). The assay is designed to detect reducing sugars generated as a result of enzyme activity by protein extracts. In order to do this the following range of reaction mixtures was set up:

Quantities shown were in microlitres (μl)

	Reaction mixtures							
CONSTITUENTS	**1a**	**1b**	**2**	**3a**	**3b**	**4**	**5**	**6**
Crude protein	62.50	62.50	0	0	0	0	0	0
Desalted protein extract	0	0	0	62.50	62.50	0	0	0
Extraction Buffer	0	0	62.50	0	0	0	0	0
0.05M acetate buffer	18.75	18.75	18.75	18.75	18.75	81.25	112.50	125.00
1mM sodium dithionite in 0.05M acetate buffer	12.50	12.50	12.50	12.50	12.50	12.50	12.50	0
1% PGURA in 0.05M acetate buffer	31.25	31.25	31.25	31.25	31.25	31.25	0	0

The reaction mixtures

All the reaction mixtures except Ib and 3b were made up completely and the incubated for a predetermined time (four or more hr.) After this time the reducing power in the mixtures was measured. RM Ib and 3b were set up without the crude extract or protein extract. These additional components were not added until after incubation and immediately before their reducing power was measured. RM Ib and 3b served as controls for RM Ia and 3a. The reducing power of RM Ia and 3a minus that in Ib and 3b respectively gave values for the reducing power which was generated as a result of incubation of substrate with protein extracts. RM 2, 4, 5 and 6 were set up to show the reducing power which was contributed by the component parts of the reaction mixtures Ia and 3a. In most experiments where this method was used RM I to 6 were all at pH 5.0.
In addition to these RM 3a, 3b, 4, 5 and 6 were made up with all the components at pH 4.5, 4.0 or 3.5.

Making up the RM

Crude extracts were made using acetate extraction buffer by either of the methods described in section 5.1 of this chapter. The desalted protein extracts were made by passing crude extract through Sephadex columns equilibrated with the appropriate buffer. (pH, 3.5, 4.0, 4.5 or 5.0 as described in section 5.2.I). The 1% PGURA solutions of different pH were made by overnight dialysis at room temperature of 10ml samples of 1% PGURA in 0.05M acetate buffer pH 5.0 against 2l. of 0.05M acetate buffer of the required pH. Sodium was added to reaction mixtures to inhibit uronic acid oxidase. This enzyme oxidises galacturonic acid. Its presence can lead to a reduction in the measured reducing power. Solutions of sodium dithionite were made by dissolving the correct amount of solid in 0.05M acetate buffer of the appropriate pH.

Measuring the reducing power

To measure the reducing power on equal volume (125µl) of low alkalinity copper reagent (the constituents of which are given below) was added to each reaction mixture. After 10 min in a boiling water bath the tubes were cooled by transfer to a cool water bath before adding 250µl of arsenomolybdate reagent (see below). The solutions were vortexed till no more bubbles evolved. Then 1ml of distilled water was added and the tubes were allowed to stand for 15min to allow a colour to develop. After this time the absorbance of 520µm was measured with a Pye Unicam double beam spectrophotometer. The reducing sugar content in each solution was estimated by comparing these absorbance values with those obtained for a series of galacturonic acid solutions. Standard solutions containing 0.1, 0.2, 0.3, 0.4 and 0.5 mg galacturonic acid per ml of buffer (acetate) were made up as

follows:-

µl of stock solution	µl of buffer	resulting concentration
0	250	0.0
50	200	0.1
100	150	0.2
150	100	0.3
200	50	0.4
250	0	0.5

The solutions were not incubated but were otherwise treated in the same way as experimental samples. The reducing sugar content produced as a result of the activity of the enzyme was calculated by subtracting the calculated values for the reducing sugar content in RM Ib and 3b from those for 6 and 3a respectively.

Reagents used in the reducing sugar assays

The arsenomolybdate reagent was made by adding 21ml of 96% sulphuric acid to a solution of 2.5g of ammonium molybdate in 450 ml of distilled water. This was followed by adding 3g of disodium hydrogen heptahydnate dissolved in 25ml of distilled water. This complete solution was incubated for 24 hr at 37^{o}C before use.

Section 7 Pigment analysis

7.1 Measurement of the chlorophyll content of *Pelargonium* petioles

This method is based on that of Holden (1965). All the analyses were performed on freshly excised or cultured 50mm *Pelargonium* petioles. For each replicate in an experiment three or four (depending on the experiment) were weighed then cut into small pieces and added to a chilled mortar and pessle on ice containing 5ml of 80% aqueous acetone, 1g of sand and 1g of calcium carbonate ($Ca.CO_3$). Calcium carbonate prevents pheophytin formation which inhibits the measurement of chlorophyll. The mixture was ground until a smooth homogenate was produced. The homogenate was filtered by suction through a Buchner funnel using glass filter paper. A further 5ml of 80% aqueous acetone was passed through the filter to wash out any residual chlorophyll (ch1) from the macerate. The filtrate was made up to exactly 10ml in a volumetric flask and then kept on ice. The absorbance of the extract was measured immediately with a Pye Unicam double beam spectrophotometer at 663 and 645 nm using quarts cuvettes. A solution of 80% acetone was used as the blank.

Knowing the fresh weight of the petioles and the absorbance of the extract, values for the chlorophyll a (chla) and chlorophyll b (chlb) concentrations in mg per g freshweight was calculated using the following formula:

$$\text{chla } (mg.g^{-1}) = \frac{12.3(A663) - 0.86(A645) \times V}{d \times 1000 \times W}$$

$$\text{chlb } (mg.\ g^{-1}) = \frac{19.3(A645) - 3.6(A663) \times V}{d \times 1000 \times W}$$

V = volume of sample in ml = 10

d = length of light path in cm = 1

W = fresh weight of tissue in replicate in g

A663 = The absorbance at 663nm

A645 = The absorbance at 645nm

Section 8 Statistical analysis

Analyses were performed according to Bailey (1959). In experiments where there were several replicates, means, standard deviations and standard errors were calculated. The T test of significance and analysis of variance were used to determine whether significant differences existed between groups of replicates which had different treatments.

Chapter 3

Chapter 3. THE RESULTS

This chapter is divided into three main sections.

(I) The morphological and structural changes which take place when *Cuscuta campestris* shoots infect *Pelargonium zonale* petioles *in vivo.*

(2) The development of an *in vitro* system to study the host/parasite interaction between *Cuscuta campestris* and *Pelargonium zonale.*

(3) An investigation into the role of certain cell wall degrading enzymes during the infection of *Pelargonium zonale* plants by *Cuscuta campestris.*

The three sections are arranged in the order in which they have been listed.

As described in chapter one an effective *in vitro* system must be representative of the *in vivo* host/parasite interaction. Therefore it was important to establish what the important morphological and structural events were *in vivo* before developing the system. It is therefore logical that the section describing the morphological and structural changes which take place *in vivo* should be presented before that describing the development of the *in vitro* system.

The investigation of the role of certain cell wall degrading enzymes in the infection of *Pelargonium zonale* plants by *Cuscuta* involves the measurement of changes in enzyme activity. If these changes in activity are to be associated with the penetration process it is important to know what morphological or structural changes coincide with them. Therefore it is logical to describe the morphological and structural changes which take place during infection before the section describing the investigation of a role of cell wall degrading enzymes in infection.

Sections 2 and 3 were investigated in parallel. Both follow on logically from section I. However they do not necessarily follow on from each other. The development of the *in vitro* system was one of the primary aims of this work which was fully achieved. The investigation of the role of cell wall degrading enzymes was closely associated with the long term aims of understanding the mechanisms by which *Cuscuta* invades the host plant and avoids triggering resistance.

It was therefore decided to put the section describing the development of the *in vitro* system before the more open ended section describing the investigation of a role of certain cell wall degrading enzymes in the infection process.

Section I The morphological and structural changes which occur during the invasion of *Pelargonium* petioles by stem tips of *Cuscuta campestris*

In order to understand the nature of the cellular interactions which take place between the host and the parasite it was first necessary to observe and record the morphological and structural changes which occur during invasion. This study provided the answers to the following questions:

(I) How is *Cuscuta campestris* adapted to its parasitic habit?

(2) How does the parasite enter the host and what is the route taken as it travels through the host tissues?

(3) Which cells of the host and parasite come into contact with each other?

(4) What types of structural disruption take place in the host as a result of parasitism?

The results presented in this section describe the changes in morphology and structure which accompany the invasion of the host by the parasite. The morphological events are described first.

I.I The morphological changes which occur during the invasion of *Pelargonium* petioles by *Cuscuta campestris*

The aim of this experiment was to identify and record the morphological changes which occurred in the parasite and host during invasion of the *Pelargonium* petioles.

Ten 50mm and seven, 60mm shoot tips of *Cuscuta campestris* were inoculated onto petioles of greenhouse *Pelargonium* plants as described in section 2.I of Ch2. Detailed observations were made after I, 3, 4, 5, 6, I0, II and I3 days and the changes in the morphology recorded.

In addition to this samples of host/parasite interaction of different ages were removed from greenhouse stock plants and prepared for Scanning Electron Microscopy (SEM) as described in Section 4.I.2. of Ch2.

The appearance of the host and parasite before inoculation

Prior to inoculation the shoot tips of *Cuscuta campestris* resembled brightly coloured wire, the stem was slender and smooth usually hooked or curved towards the apex (Fig I.Ia) and the leaves were reduced to scales.

The *Pelargonium* petiole was approximately cylindrical (diameter of approximately 2mm). green and covered in many colourless hairs (see Fig I.Ib).

Changes in the appearance of the host and parasite prior to and during invasion of the host

During the establishment of the parasite on the host four major morphological changes

Fig. I.Ia. The apical portion of a *Cuscuta campestris* shoot. A side branch (SBR) is seen arising from a leaf axil. Scale = 5mm.▼

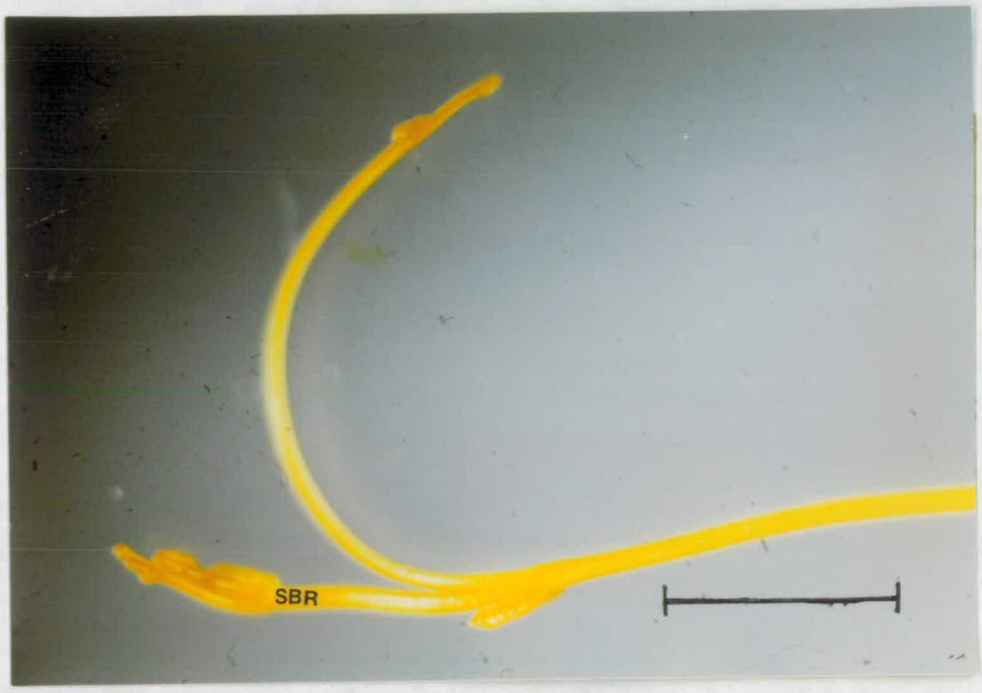
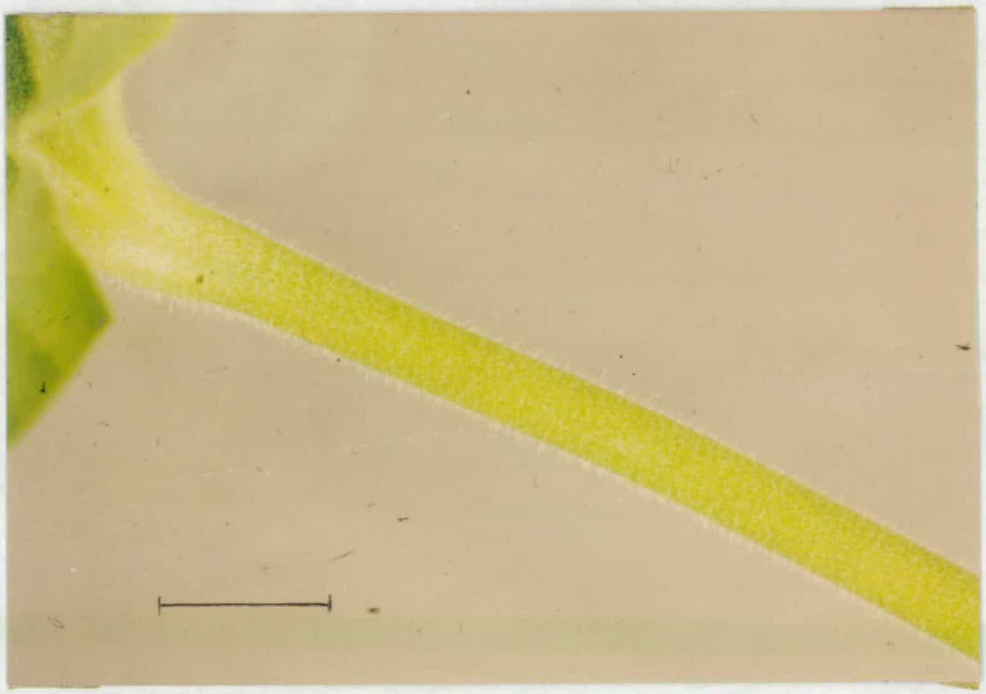


Fig. I.Ib. A *Pelargonium zonale* petiole. The petiole is green in colour and covered in many colourless hairs. Scale = 5mm. ▼



were observed:

(I) The formation of tight coils around the host (see Fig I.Ic).

(2) The development of swellings on the concave side of the tight coils (see Fig I.Id).

(3) The appearance of papillate appendages over the surface of the tightly coiled region (see Fig I.Ih).

(4) Rapid extension of the shoot tip.

These changes always occurred in the following order:

(I) Tight coils were formed around the host by extension of the shoot apex of the parasite. The coiling was always in an anti-clockwise direction. In this experiment coiling occurred within 24 hr of inoculation for all the shoot tips. After coiling the shoot apex did not extend any further. This was shown by recording the mean number of coils made around the host (the number of complete circumferences of the petiole) for each shoot tip. The mean number of complete circumferences the shoot tips made when they formed tight coils around the host was recorded after the first day and then for a further 6 days and then after I0 days. The results are seen in Fig I.If. The mean number of circumferences after I day for 50mm tips was I.0. This increased to I.2 after 3 days and I.22 after 4 days. The mean number then decreased to I.I5 after 5 days and I.I7 after 6. The mean number of circumferences were the same after I0 days as after 6 days. The mean number of circumferences made by 60mm shoot tips were greater than those made by 50mm shoot tips on all the days that measurements were taken. The mean number after I day was I.32 which increased to I.4 and I.45 after 3 and 4 days. The mean number then fell to I.25 after 5 days and then rose again to I.35 after 6 days. The mean number after I0 days was the same as for 6 days. These measurements showed that the majority of coiling was confined to the first 24hr with small increases in its length up to 4 days.

(2) Within 3 days of inoculation areas of swelling appeared all along the concave surface of the tight coiled region of the shoot tips (Fig I.1d). These swellings named prehaustoria by other workers, are the points from which the parasite invades the host. Figs I.Ic and 1.1d are SEM micrographs of different stages in the development of these prehaustoria. The prehaustoria first became obvious when islands of cells on the concave surface of the coiled region appeared to enlarge while those surrounding them did not. When viewed from the side the superficial cells of the prehaustoria which closely adhere to the surface of the host were found to become much lobed (see Fig I.Ig) when they were in contact with a host. In this example the prehaustorium was against another *Cuscuta* stem. When the host was a foreign plant the effect was the same.

(3) Five days from inoculation papillate appendages appeared over the surface of the tight coiled regions of the shoot tips. When viewed with the naked eye (see stages 4 and 5 of Fig

Fig. I.Ic. A *Cuscuta campestris* (CC) shoot has formed light coils around a *Pelargonium zonale* petiole. (PZ). Scale = 5mm. ▼

Fig. I.Id. An SEM micrograph showing a very early stage of the development of prehaustoria. Regions of raised cells (RC) are seen along the concave side of the *Cuscuta campestris* stem. ▼

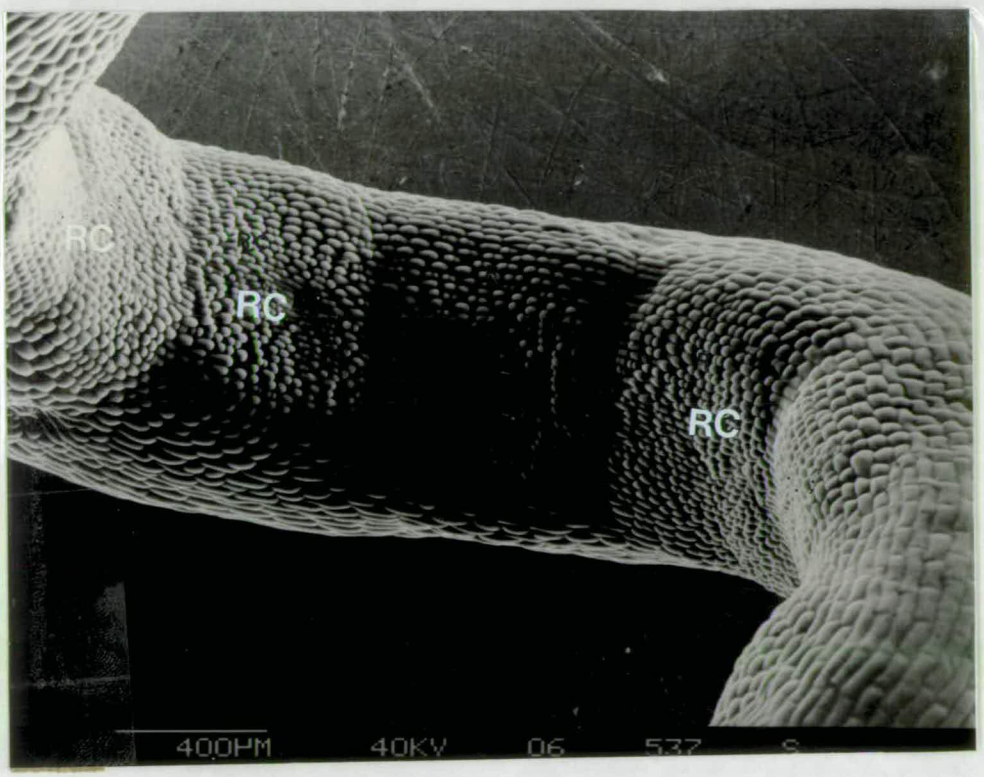


Fig. I.Ie. An SEM micrograph showing a more advanced stage of prehaustorial development. The areas of raised cells now appear as distinct organs on the concave side of the *Cuscuta campestris* stem. ▼

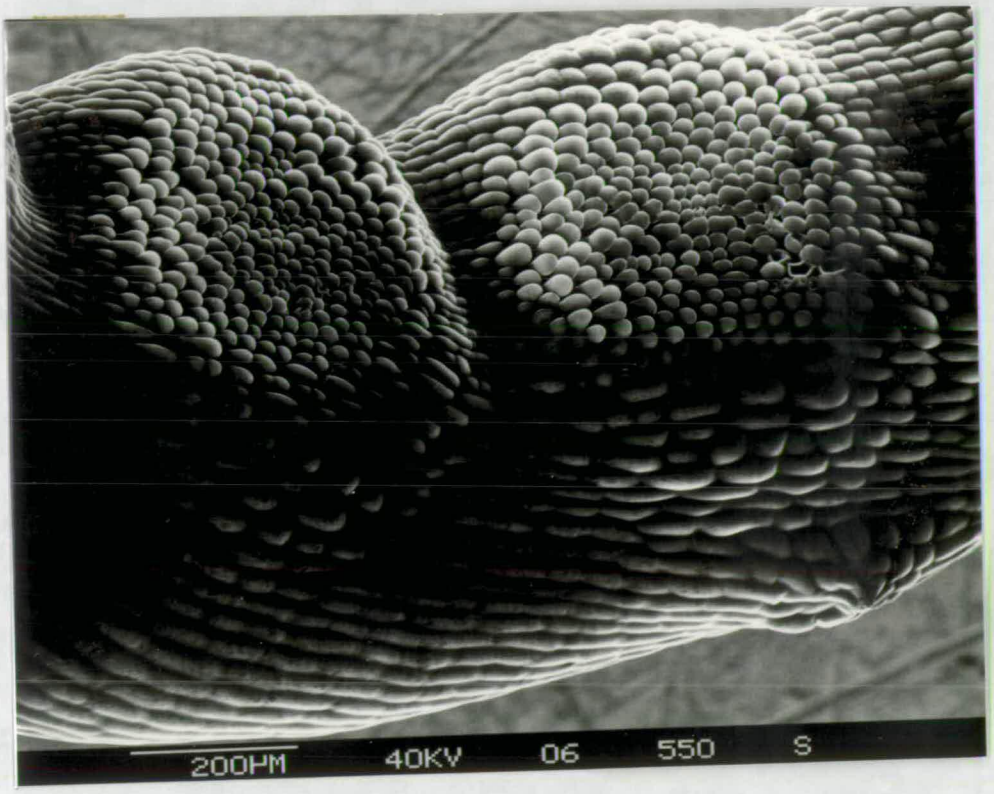


Fig .1.1.f. The mean number of complete circumferences made by 50 and 60mm shoot tips of *Cuscuta campestris* which were inoculated onto petioles of greenhouse *Pelargonium* plants (I) = standard error bars

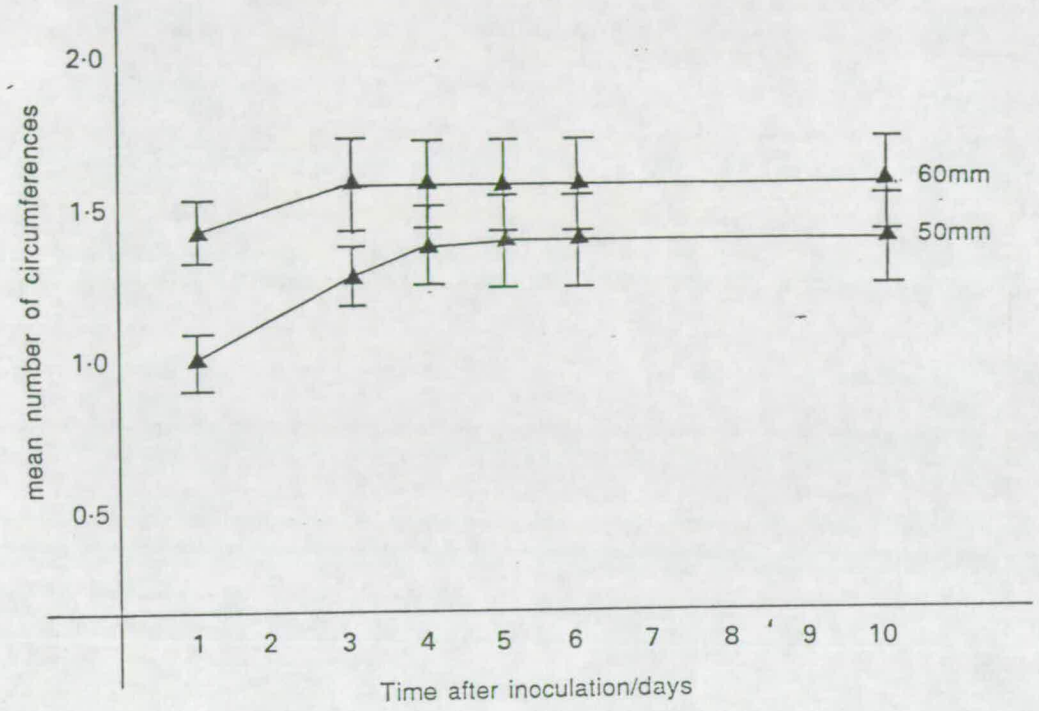
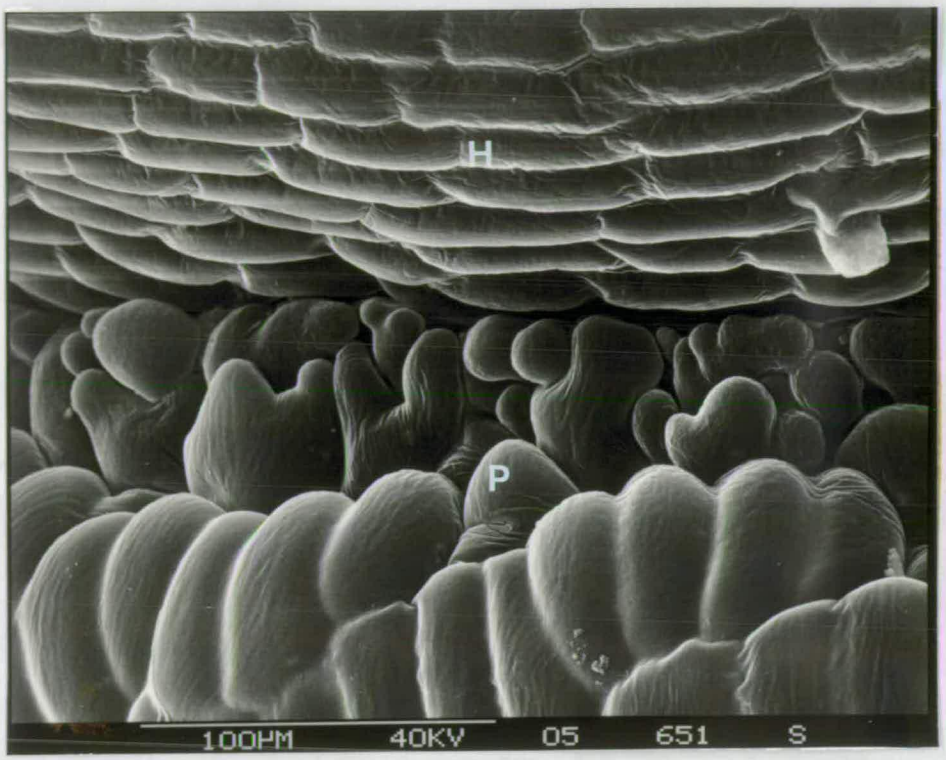


Fig.I.Ig. SEM micrograph showing the lobing of the superficial cells of a *Cuscuta campestris* (P) prehaustorium in contact with the host (H).▼



l.ll) the appendages appear as raised cones on the stem surface. When observed using the SEM they are seen to be multicellular structures (see Fig l.lh). The conical shape appears to be due to the elongation of several cells, away from the surface of the stem. The top of the cone formed is spanned by 2 sausage shaped cells (SSC). These are similar in appearance to typical guard cells of stomata. In between these cells an opening can be seen (see P in Fig l.lh). By observing from direcly over the papillate appendage at higher magnification it was possible to see the opening was connected to a chamber beneath (see Fig l.li). These papillate appendages were found only on the coiled regions of the parasite stem.

(4) From 6 days after inoculation onwards the region of the shoot tip from the last part of the tightly coiled region to the tip began to extend rapidly. This region was named the 'extending region'. The size of each extending region was measured on days 6, l0, ll and l3 post inoculation. The results are shown in Table 1.1j On day 6 the extending region of shoot tips which had been 50mm in total length when inoculated ranged between 2 and l5mm with a mean of 5.2. By day l0 the length of the extending regions had increased. They ranged between 8 and 44mm in length with a mean of 20.4mm. All the extending regions were longer the following day ranging between ll and 68mm with a mean of 37.3mm. Finally on day l3 these extending regions ranged between 30 and l90mm in length with a mean of 93.lmm.

The extending region of shoot tips which had been 60mm in total length when they were inoculated extended in a similar manner with time. Their extending regions on days 6 ranged between 6 and 8mm long with a mean of 6.8. By day l0 the extending regions had lengths between l9 and 58mm with a mean of 37.0mm. On day ll the extending regions had further increased in size measuring between 37 and 90mm with a mean of 62.6mm. Further increases in length occurred in all the extending regions by day l3. Their lengths on this day ranged between 78 and l38mm with a mean of ll4.6mm.

The rate at which these extending regions increased in length was calculated and the results are shown in Table l.lk. The results showed that the rate of extension of the extending regions of shoot tips which were either 50 or 60mm in total length when first inoculated, increased with time post inoculation up to and beyond ll days. Of the extending region of shoot tips which had been 50mm in total length when inoculated between 6 and l0days was 5.75mm per day. This increased to l5.l7mm per day between l0 and lldays with a further increase to 23.75mm per days between ll and l3 days. The mean extension rates of the extending regions of shoot tips which had been 60mm when first inoculated were larger than those for shoot tips which had been originally 50mm. Between 6 and l0days the mean extension rate was 7.5mm per days. This increased to 24.60 and 27.l2mm per day between l0 and ll and ll and l3 days respectively.

Fig. I.Ii. SEM micrograph showing the pore (P) of a papillate appendage in between two sausage shaped cells (SSC) which appears to lead to some sort of space or chamber. ▼

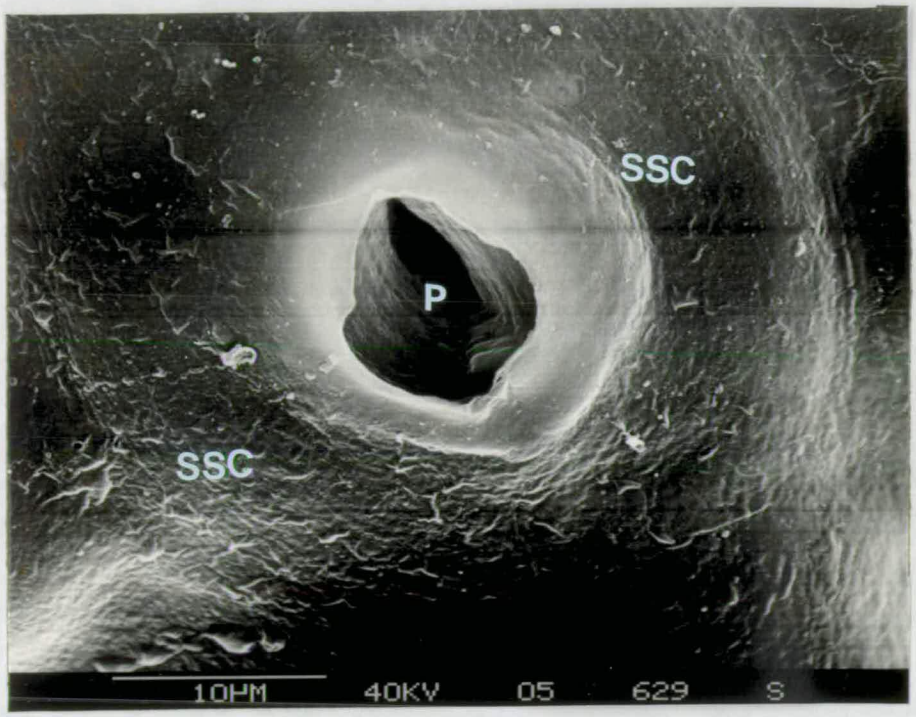


Fig. I.Ih. SEM micrograph showing a single papillate appendage on the surface of a coiled region of *Cuscuta campestris*. The papillate appendage consists of two sausage shaped cells (SSC) joined at their ends but separated along their lengths by a pore (P). These cells are held above the level of the rest of the stem by a ring of supporting cells. ▼

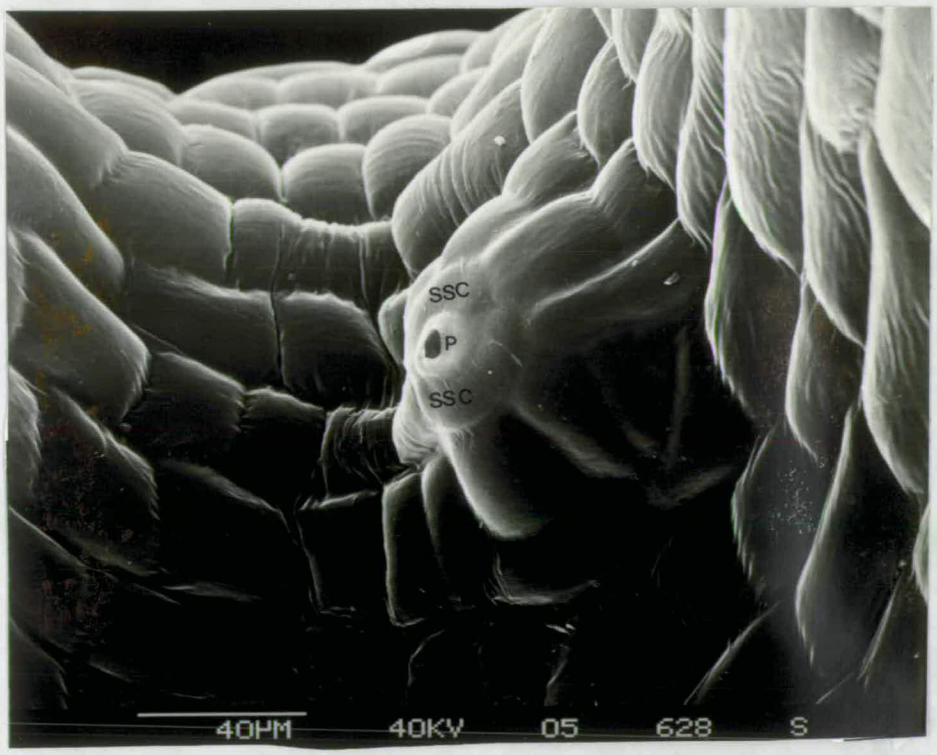


Table 1.1j. The length of the region from the last coils to the apices of *Cuscuta campestris* shoot tips (originally 50 and 60 mm) 6, 10, 11 and 13 days post inoculation.

Original length of shoot tip (mm)	replicate	Length (mm)			
		6days	10days	11days	13days
50	1	-	-	-	-
	2	2	44	68	115
	3	3	22	41	100
	4	3	40	51	102
	5	-	-	-	-
	6	3	17	30	55
	7	15	-	-	190
	8	-	12	23	60
	9	5	8	11	30
	10	-	-	-	-
60	1	6	32	-	110
	2	6	19	37	78
	3	8	42	67	136
	4	-	-	-	-
	5	6	58	90	138
	6	7	35	60	124
	7	8	36	59	102

Table 1.1k. The rate of extension of the region from the last coil to the apex for 50 and 60mm *Cuscuta* shoot tips inoculated on to *Pelargonium* petioles.

original total length	Days after inoculation	mean rate of extension	standard error se
50	6-10	5.75	± 1.81
	10-11	13.50	± 2.96
	11-13	20.67	± 3.37
60	6-10	7.54	± 1.30
	10-11	26.60	± 3.08
	11-13	25.50	± 3.47

When the extending region began to extend the shoot tips came into contact with other petioles of the *Pelargonium* plant. This resulted in coiling, prehaustorial formation, appearance of papillate appendages and tip advance, and so the cycle continues.

Other visible changes

In addition to the four main morphological changes which occurred at specific points during invasion there were other changes to the tightly coiled regions. These included changes in colour.

Colour changes

Tight coiled regions exhibiting the different colours were collected together and photographed (see Fig.I.Il). Shoot tips were found to be in one of four colour classes, orange/yellow, yellow, yellow/green or green. The colour changes of the 50 and 60mm shoot tips were monitored over a I0 day period following inoculation. The proportions of replicates exhibiting each colour class were recorded on days 0, I, 3, 4, 5, 6 and I0. The results are displayed in Fig I.Im and n. When first inoculated shoot tips were either orange/yellow or yellow. The colour changes of the 2 types of tips showed similar trends but differed in detail. One day after inoculation 7I% of the 50mm shoot tips were still orange/yellow, I4.5% yellow and I4.5 yellow/green (see Fig I.Im). However, by three days, only I4.5% were orange/yellow and none were yellow. The remainder were green and yellow/green (I4.5 and 7I% respectively). By the fourth day all the coiled shoot tips were yellow/green. On the fifth day I4.5% were green and 85.5% were yellow/green. After 6 days 14.5% of the coiled stems had become yellow/orange again, while 14.5% were green and 71% yellow/green. After I0 days the number which were yellow/green had decreased to 43%. None of the shoot tips were green or yellow and the remainder (57%) were orange/yellow.

The pattern of the changes in colour for 60mm shoot tips (Fig I.In) showed similar trends to 50mm shoot tips but with some differences. After I day 50% of the tips were still yellow, 33.3% orange/yellow and I6.7% yellow/green. By 3 days all the shoot tips had become yellow/green. After 4 and 5 days the proportion of yellow/green shoot tips had decreased to 33.3%. The proportion which were green increased to 66.7%. After 6 days the proportion which were green decreased to zero as all the shoot tips became yellow/green. Finally by I0 days all the shoot tips were orange/yellow. These two experiments showed that the colour of shoot tips does change as they mount an infection on the host plant. During this process the proportion of green pigmentation in the stem appears to increase greatly. This green colour disappears again as the parasitism develops. One day old coiled regions which bear prehaustoria are yellow/green. The

Fig. I.I.I The different colours displayed by the tight coiled regions of a population *Cuscuta campestris* shoot tips during different stages of the infection of *Pelargonium* petioles.▼ ▼

orange/ yellow yellow yellow/ green green.

Fig. I.Im. The change in the proportions of tight coiled regions of a population of *Cuscuta campestris* shoot tips which appeared particular colours with time after inoculation. These shoot tips were 50mm when inoculated. (●) yellow/orange, (□) yellow, (▲) yellow/green and (○) green

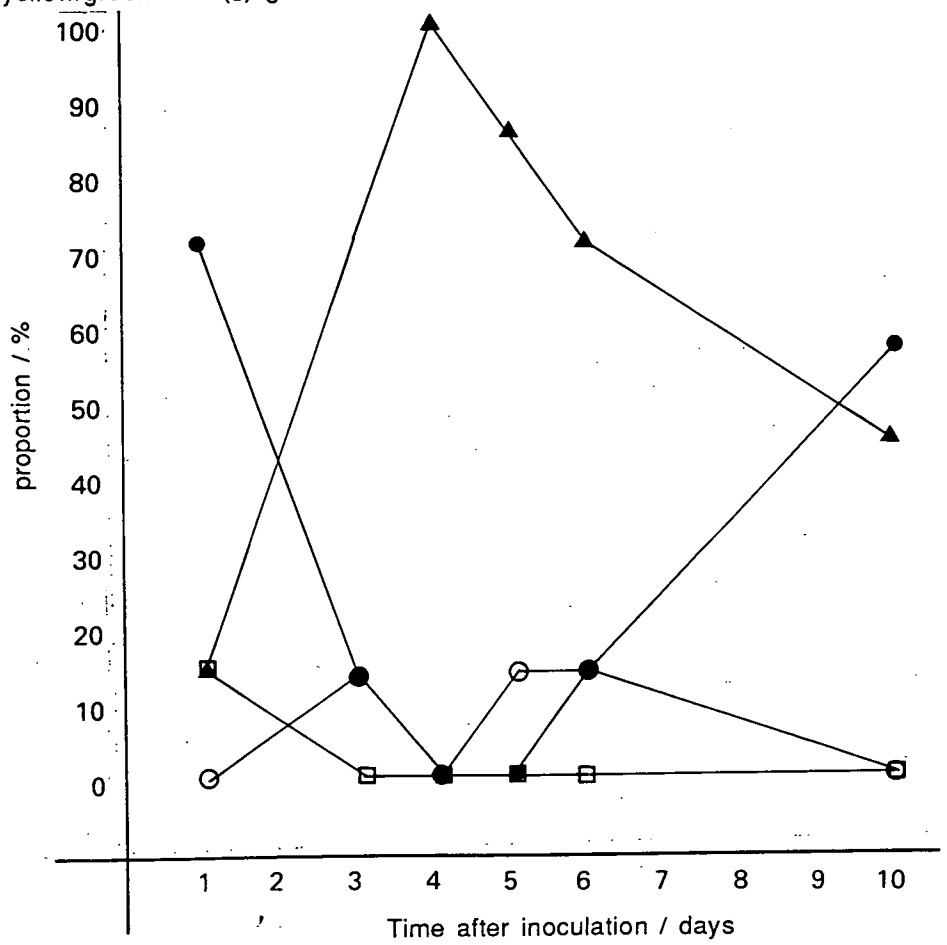
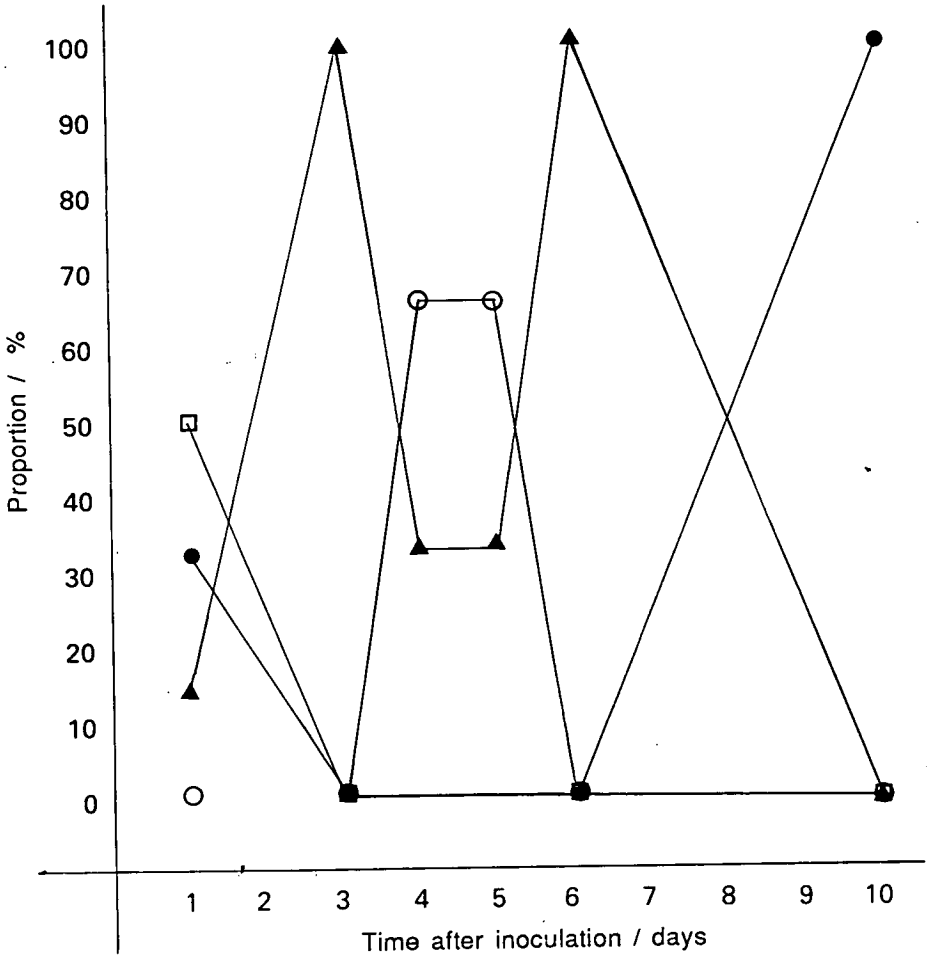


Fig.I.In. The change in the proportions of tight coiled regions of a population of *Cuscuta campestris* shoot tips which appeared particular colours with time after inoculation. These shoot tips were 60mm when inoculated. (●) yellow/orange, (□) yellow, (▲) yellow/ green and (○) green.



mature coiled regions were found to be the most deeply orange/yellow pigmented parts of the plant. In adition to the changes in colour the tight coiled region of the parasite are observed to change in diameter with time during invasion.

Changes in the diameter of the tightly coiled region

Soon after coiling the tightly coiled region was found to swell relative to the rest of the shoot tip. The uncoiled cut end of the shoot tip in contrast becomes shrivelled and brown. In an attempt to quantify the changes in the diameter of the parasite stem in the tightly coiled region a 'Relative Turgidity' (RT) scale was created. This semi-quantitative scale was designed to indicate the extent to which the diameter of the stem of the parasite changed with time. Fig I.Io shows how the diameter of the stem in the coiled region varies. The range of diameters were separated into six size groups numbered from 0 to 5. During observations the diameters of the stems of the parasite were estimated by reference to this scale. In general when first inoculated the coiled regions of the parasite have an RT of 2. If the parasite succeeded in penetrating the host the RT was found to increase with time. If the parasite did not succeed the RT gradually decreased with time. When the RT was O the coiled region had become completely dried out.

The RT of 50 and 60mm *Cuscuta* shoot tips which were inoculated onto *Pelargonium* petioles were monitored over a 10 day period. Recordings were made after 0, 4, 5, 6 and I0 days.

The results are shown in Fig I.Ip and q. All freshly inoculated shoot tips whether 50 or 60mm had a RT of 2. By 4 days I0% of the 50mm shoot tips which had been inoculated onto the host had an RT of I. This meant that the tips were drying out. None of the shoot tips had an RT of 2. The majority of the shoot tips had an increase in RT. Fifty per cent of the tips had a coiled region with an RT of 5. In addition to this 20% had an RT of 3 and 20% one of 4. After 5 days 40% had an RT of 5 while 30% had an RT of 4. The proportion with an RT of 3 had decreased to I0%. In addition I0% had an RT of 0 and I0% an RT of I. By I0 days the proportion which had an RT of 5 had increased to 60%. The remaining shoot tips had RTs of 4 (I0%), 3 (I0%), 2 (I0%) and I (I0%).

The pattern of change in the RT of the coiled region for the 60mm tips was slightly different (see Fig I.Iq). After 4 days I6% of the shoot tips had an RT of I. The rest had an RT of either 4(42%) or 5(42%). The following day the proportion which had an RT of I was still I6%. However the proportion which had an RT of 4 decreased to 28% and those with an RT of 5 had increased to 66%. After 6 days the proportions of tightly coiled regions with particular RTs was the same as after 4 days. By I0 days I6% of the shoot tips still had an RT of I. The rest (84%) had an RT of 5. All the shoot tips which proceeded to tip advance stage had a relative turgidity of 5. The non coiled parts of the

Fig.I.Io The semi-quantitative relative turgidity scale (RT) used to express the degree of swelling of the tight coiled region of *Cuscuta campestris* shoot tips following inoculation. A RT of O represents a completely dried out shoot tip, a RT of 5 represents a fully swollen coiled region.▼

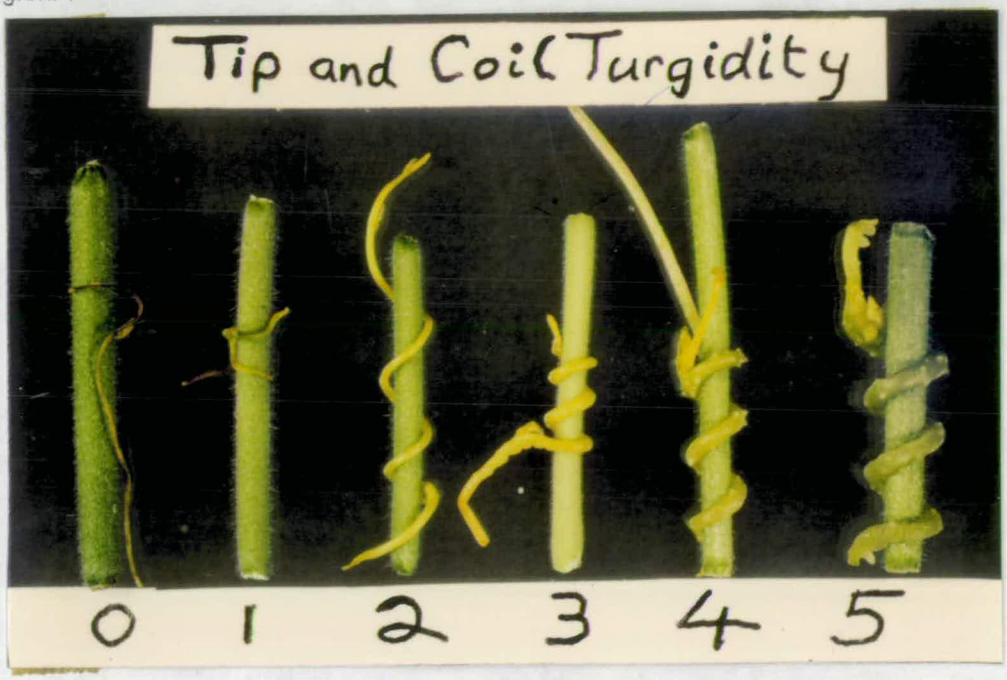


Fig. I.Ip. The change in the proportions of tight coiled regions of a population of *Cuscuta campestris* shoot tips with a particular RT with time after inoculation. These shoot tips were 50mm when inoculated. (△) = 1, (■) = 2, (●) = 3, (○) =4 and (▲) =5. □

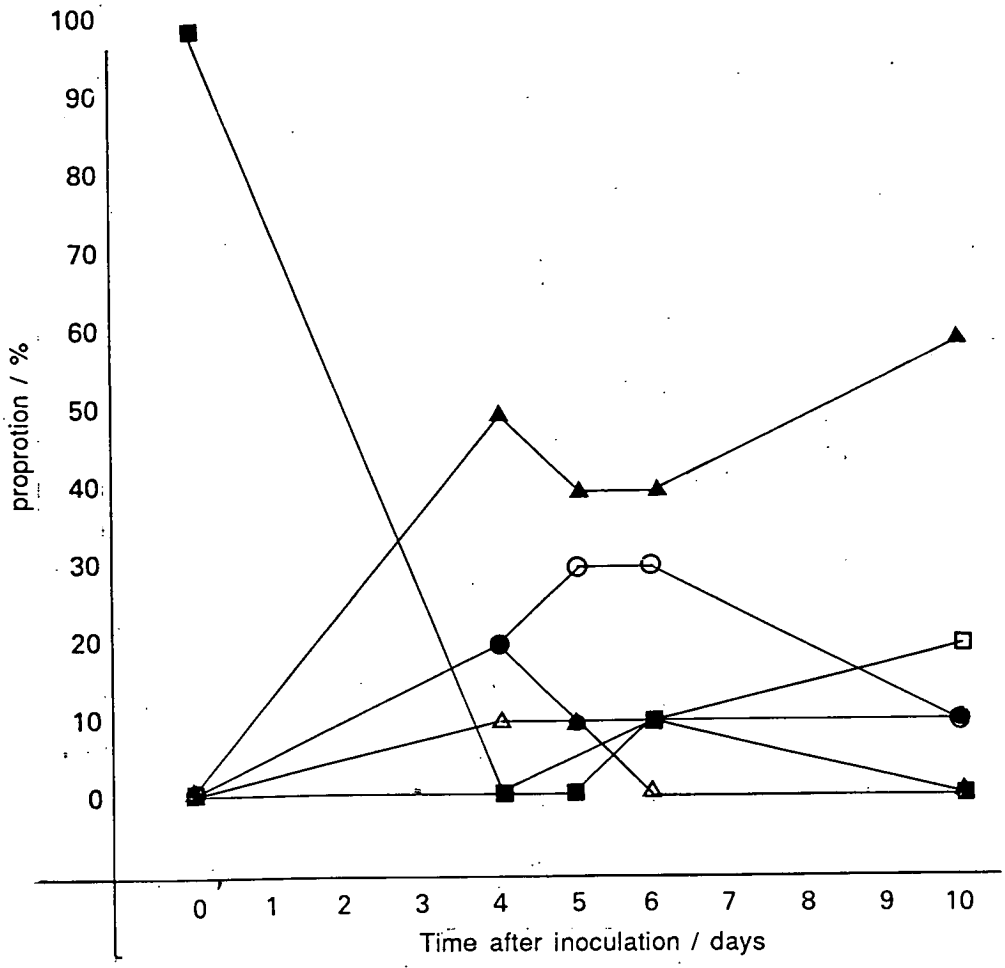
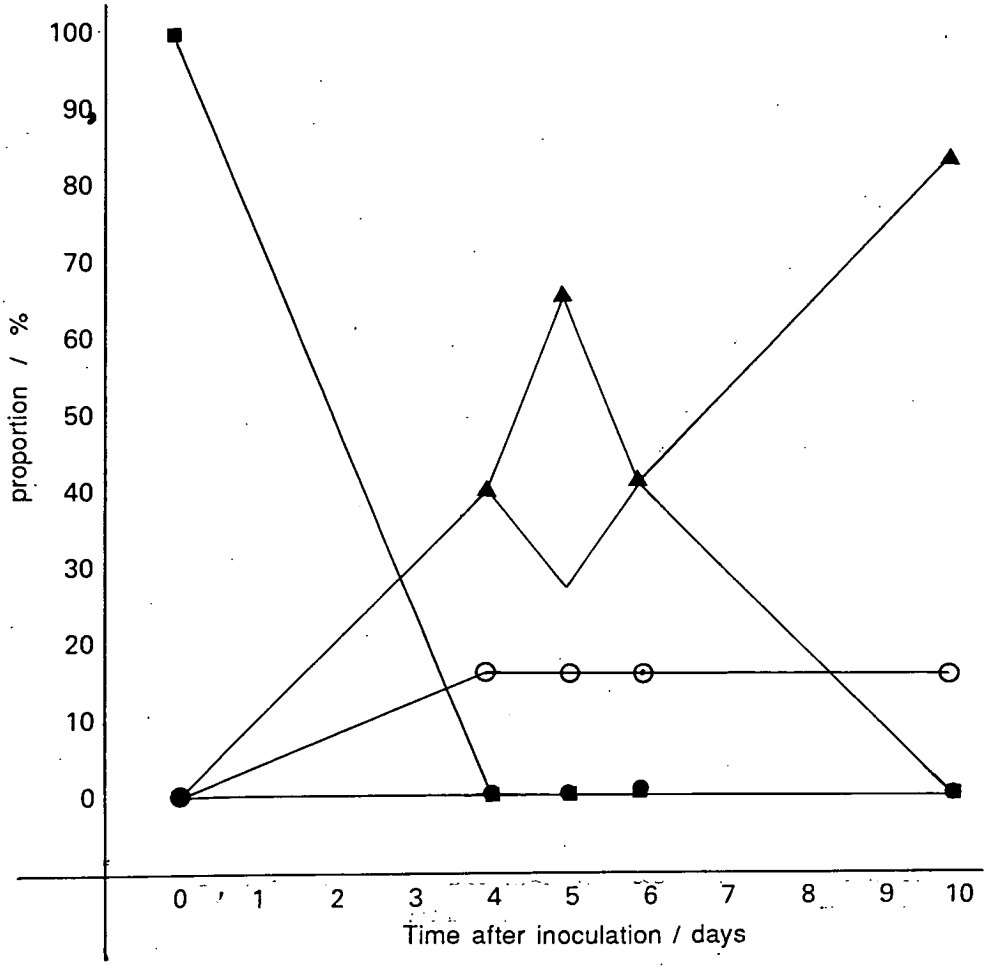


Fig. I.Iq. The change in the proportions of tight coiled regions of a population of shoot tips with a particular RT with time after inoculation. These shoot tips were 60mm when inoculated. (Δ) = 1, (■) = 2, (●) =3, (○) =4 and (▲) =5. □



shoot tips did not increase in diameter during the invasion of the host. They usually became shrivelled and discoloured.

Changes in the appearance of the host

The host petiole did not appear to change greatly during the parasitism by *Cuscuta.* However some of the petioles became swelled in the area directly under coiled stem of the parasite. The frequency with which this swelling occurred is detailed in Fig 11r For 50mm shoot tips there was visible swelling in 3 out of the 7 shoot tips which proceeded to the tip advance stage. Of these the swelling was first observed in 2 of them, 6 days after inoculation. For 60mm shoot tips 2 out of the 6 which had reached the tip advance stage coiled around petioles which were seen to swell slightly 6 days after inoculation. The parasitised petioles remained green showing no signs of senescence or discolouration.

This experiment showed the timing and order of the morphological events which occurred to the host and parasite during invasion of petioles of *Pelargonium zonale* by shoot tips of *Cuscuta campestris.*

(a) Within twenty-four hours of inoculation the shoot tips coil around the host of the plant provided they are in contact and correctly orientated. Little further length is added to the coiled region after the first 24hr and none after four days.

(b) Prehaustoria, the raised pad-like structures from which the haustorium (the feeding organ) proper arises, develop within three days after inoculation. The cells in direct contact with the host become much lobed.

(c) Papillate appendages form over the surface of the coiled regions of the parasite. They first appear after approximately five days post inoculation. SEM work showed them to be multicellular structures. They are raised cones formed by adjoining cells which elongate at right angles to the surface of the stem. They are capped by two sausage-shaped cells. In between these two cells is a pore or opening which allows air direct contact with the sub- epidermal layers. The opening appears to be connected to a sub-epidermal chamber. The sausage-shaped cells resemble the guard cells of stomata.

(d) The shoot apex remained dormant after coiling until approximately six days post inoculation. Then it began to extend rapidly. The rate of extension per day increased each time measurements were taken beyond II days post inoculation. This rapid extension and branching of the apex allowed the shoot tip to invade other petioles from the same or other plants.

In addition to these changes the coiled regions were found to change colour during invasion of the host. The shoot tips began infection as orange/yellow. Following inoculation and coiling they became increasingly more yellow and then green. The process is then reversed and the colour proceeds from green back to orange/yellow via

Table 1.1r. The incidence of host swelling during parasitism of *Pelargonium zonale* petioles by *Cuscuta campestris* shoot tips, 50mm or 60mm in length. (d) = days.

Length of shoot tip (mm)	number of coiled shoot tips	number of petioles swelling	time of appearance of swelling
50	7	3	2 at 6d 1 at 10d
60	6	2	2 at 6d

yellow/green and yellow. The coiled region of the parasite becomes swollen as the parasite becomes more developed on the host.

Certain events were found to coincide during the development of the parasite on the host. For example the development of the papillate appendages coincided with the largest amount of visible green pigmentation in the parasite stem.

The host petioles remained green throughout the experimental period of 10 days. However a variable proportion were found to swell in the region directly under the tightly coiled parasite stem. Swelling was not evident till six days after inoculation.

This experiment has shown the timing and order of changes in the external morphology which occur during the invasion of *Pelargonium* petioles by *Cuscuta campestris* shoot tips. It is clear that the host and parasite responded to each other at the multicellular level. In order to study the cellular interactions which take place at the interface between the two organisms it is necessary to observe the interaction at the microscopic and sub microscopic level. The results of such a study are presented in the following section.

I.2 The sequence of structural changes which occur during the invasion of *Pelargonium* petioles by shoot tips of *Cuscuta campestris*

The aim of this experiment was to provide a detailed record of the structural changes which occur as the parasite invades the host tissues and to observe the host/parasite interface at different stages of the infection process.

A population of *Cuscuta campestris* shoot tips 150mm in length were inoculated on to petioles of greenhouse *Pelargonium* plants as described in section 2.1 of Ch2. Shoot tips this length were chosen to ensure that there were enough reserves for the parasite to mount an infection. Each shoot tip was given a code number. After 24hr each shoot tip was inspected to see if it had formed tight coils around the petiole. Each day thereafter for 13 days, three host/parasite interactions were harvested, fixed, dehydrated and embedded as described in section 4.1.1 of Ch2. Sections (0.5µm) of the resin blocks were cut and stained as described in section 4.3.1 of Ch2. Uncoiled shoot tips and uninfected petioles were also fixed, dehydrated and embedded in the same way. After viewing sections of host/parasite interactions from 1-14 day old a photographic record of the structural changes was compiled. These changes are described below and illustrated in Fig. 1.2a-s.

Structure of the host and parasite before Inoculation

A cross section of the uncoiled parasite (Fig 1.2a) reveal that its cells are approximately hexagonal with few air spaces between them. In another example a cross

Fig. I.2a. Transverse section through an uncoiled *Cuscuta campestris* stem showing many cells containing starch grains (SG). No stomata are visible. Technical details : Light microscope, 1µm thick, resin embedded, Toluidine blue stained, magnification X 95 .▼

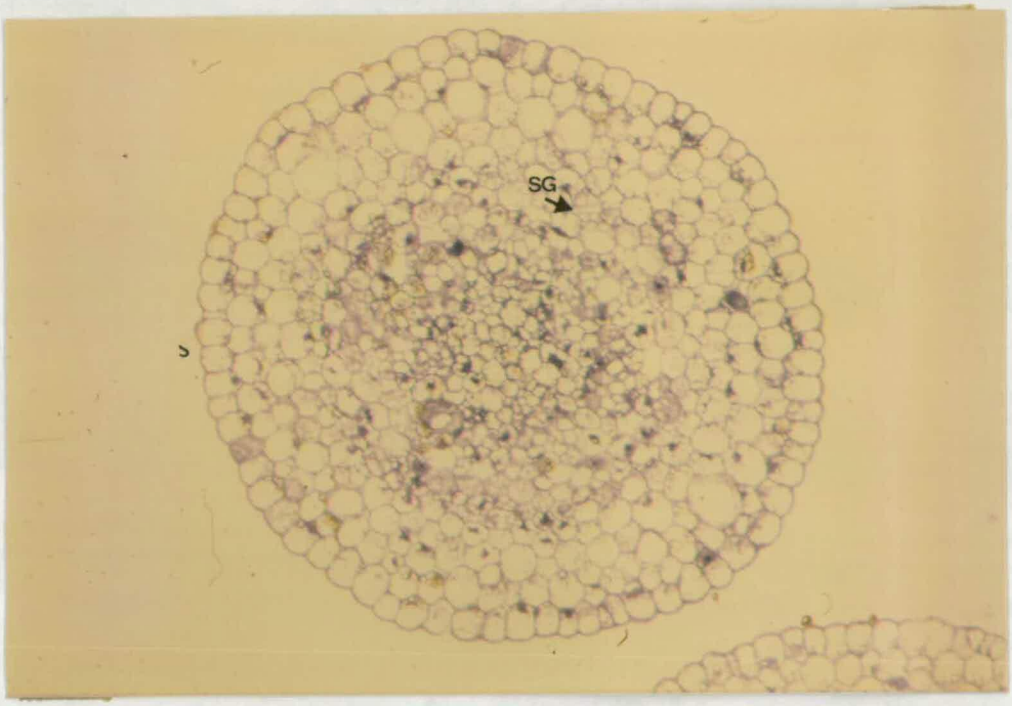
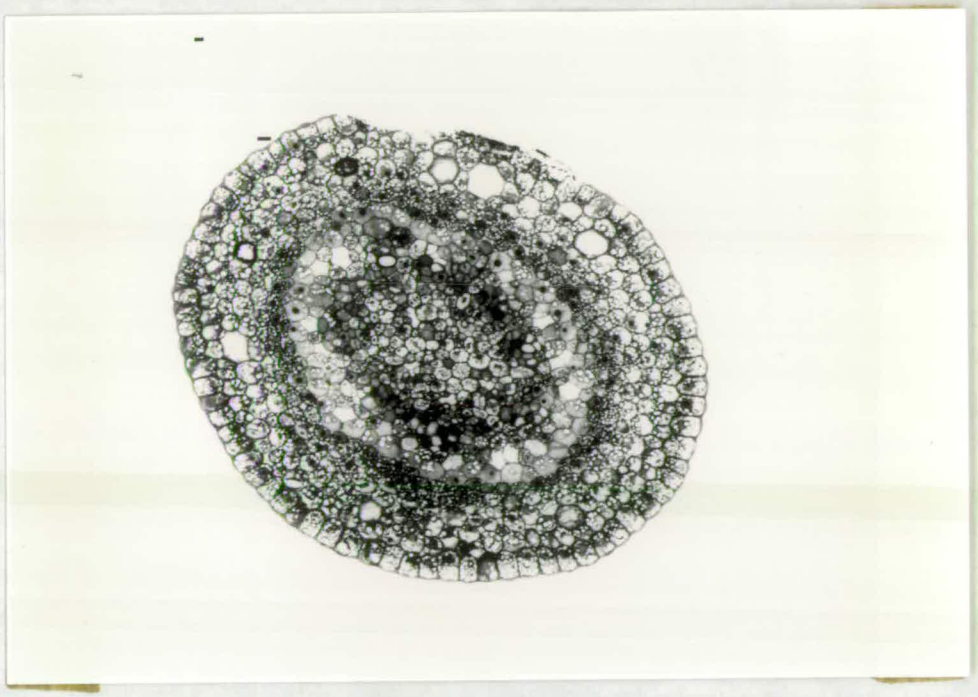


Fig. I.2b. Transverse section through another uncoiled *Cuscuta campestris* stem showing the variation in the cyloplasmic density and amount of stored starch in different parts of the plant. Technical details : Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification x 76 .▼



section from another part of an uncoiled stem large numbers of cells are shown to have deeply stained cytoplasm containing numerous starch grains (see Fig I.2b). In Fig I.2a these densely cytoplasmic cells are concentrated towards the centre of the stem. Whereas in Fig I.2b all the cells are deeply stained.

Fig.I.2c shows a cross section of a *Pelargonium* petiole. This cross section was actually taken from an *in vitro* cultured petiole. The basic structure in *in vivo* and *in vitro petioles* is the same. The T.S. of the petiole shows it to be roughly cylindrical with one side being slightly flattened. The very centre of the petiole is made up of a large vascular bundle (VB). Surrounding this are several layers of parenchyma (P) within a 2 celled layer of sclerenchyma (Sc). Around the inner circumference of this sclerenchyma layer are four additional large vascular bundles (LVB). The one nearest the flattened edge of the petiole is elongated laterally. The other three were roughly circular. In between the large vascular bundles are several small vascular bundles (SVB). Outside the sclerenchyma layer are 3 or 4 layers of parenchymatous cells surrounded by two layers of cells with thickened walls. The outer layer of these is the epidermis. Many of the cells of the epidermis are extended to form colourless hairs (H).

The structural changes which occur from the time of coiling of the parasite to the penetration of the host.

Within 24hr of inoculation shoot tips have coiled around the petiole. Transverse sections of coiled stem of this age reveal changes in the distribution of staining intensity (see Fig I.2d) compared to that of uncoiled stems (see Fig I.2a). The cells on the side of the parasite which are in contact with the host contain more starch grains and have a denser cytoplasm than those cells on the opposite side. A transverse section of the host with the parasite coiled round it (cutting through the parasite longitudinally) shows the same asymmetrical distribution of deeply stained cells as the transverse section (see Fig I.2f). The average cross-sectional area of the three layers of cells nearest the parasite in contact with the host was compared with that for the three layers of cells at the greatest distance from the host. The areas and lengths of the cells were measured in arbitrary units using an Apple steriometry package and an Apple graphics tablet. Fifty one cells were measured on the inner surface and thirty two on the outer surface. The results are presented in Fig I.2e. The mean area of the cells in the three layers of cell nearest to the host was 0.0133 while that for the outer three layers was 0.0318. The mean cell length in arbitrary units of the inner cells was 0.488, and that of the outer cells 0.779. The mean area of cells in the outer three layers is larger than that for the inner 3 layers. The outer layers of cells have also have a longer mean length than the inner layer of cells.

At this stage the only point of contact between host and parasite was the complete inner

Fig. I.2c. Transverse section of a cultured *Pelargonium* petiole. Several stomata (St) and hair cells
(HC) are seen along the epidermis. The five large and three small vascular bundles (Vb) in the cortex of the petiole are surrounded by a double layer of sclerechyma (Sc). Technical details : Light microscope 0.5 µm thick, resin embedded, Toluidine blue stained, magnification X 50 ▼

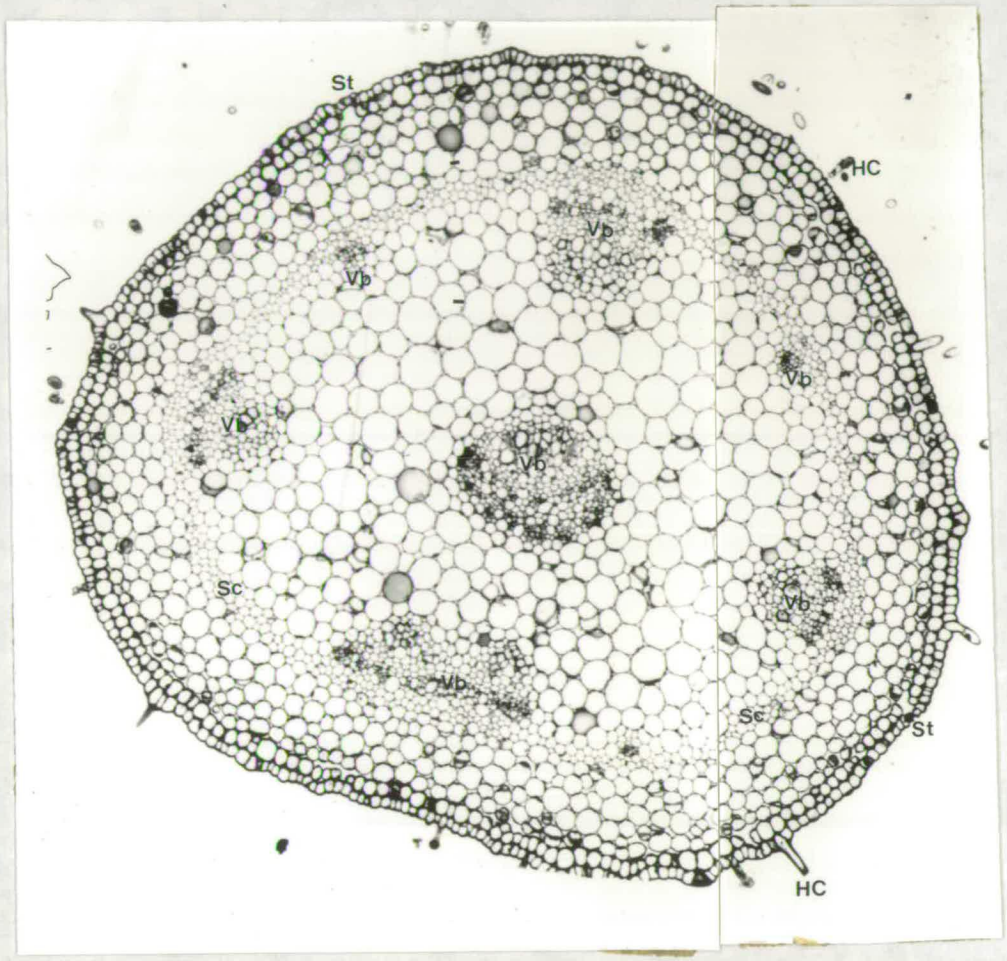


Fig. I.2d. Transverse section through a coiled part of a *Cuscuta campestris* stem 24hr after being inoculated onto a *Pelargonium* petiole. The part of the parasite stem closest to the host is seen to retain the dense cytoplasmic staining and starch grains found in all parts of the uncoiled stem. Cells at the opposite side of the stem have lost these characteristics. Technical details : Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification X300. ▼

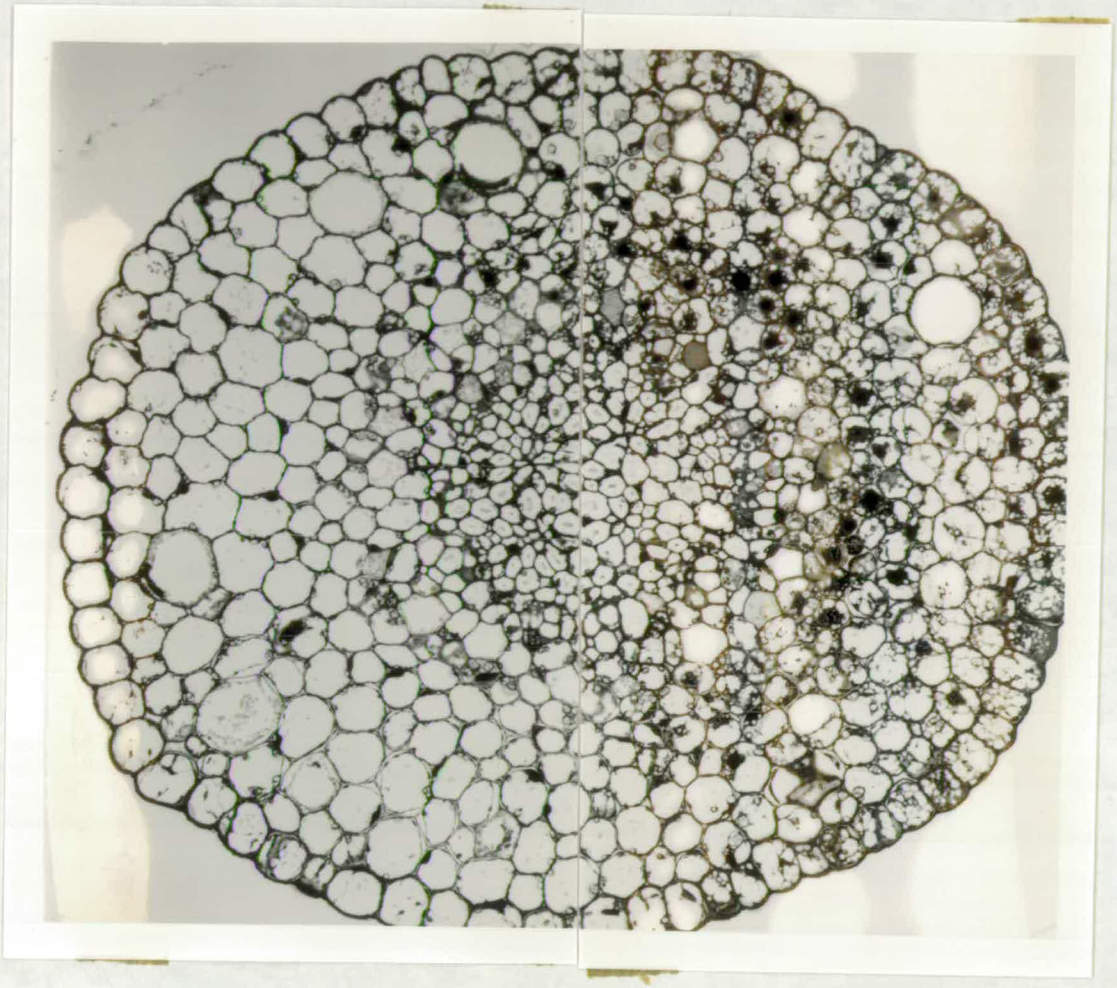


Table 1.2e The mean relative areas and lengths of the three outer and three inner layers of a parasite stem twenty-four hours after inoculation (see Fig1.2f) in arbitary units.

surface	number of cells measured	mean relative area	se	mean relative length	se
inner	51	0.0133	8.178×10^{-4}	0.4879	0.0177
outer	32	0.0318	2.92×10^{-3}	0.7793	0.0464

Fig. I.2f. Transverse section through part of the coiled region of *Cuscuta campestris* 24hr after being inoculated onto a *Pelargonium* petiole. This section shows the different staining properties of cells of the parasite on the concave and convex side of the coiled region. Technical details : Light microscope, 0.5µm thick, resin embedded, magnification X 50

side of the tight coiled region of the parasite with the epidermis of the host.

Three days after inoculation a series of prehaustoria developed along the length of the stem of *Cuscuta* (see Fig I.2g). Cross-sections through the coiled parasite show how various cell layers were modified during the development of the prehaustoria. The sub-epidermal cells have become elongated at right angles to the length of the parasite stem (see SEL Fig I.2g). The epidermal cells are also elongated in the direction of the host. The ends of the cells in contact with the host have become repeatedly lobed (see ES Fig I.2h). The tips of the lobed cells appeared to have exuded something which enabled the surface of the prehaustoria to perfectly mould to the surface of the host. This is shown in Fig I.2h. At the same time in the centre of each prehaustorium there is a deeply-stained group of small cells (see Ha Fig I.2g). These cells appear to be an early stage in the development of the absorptive feeding organ (the haustorium) which grows towards and penetrates the host. Figure I.2i is a section of a later stage of the host/parasite interaction. Here the haustorium has become much larger and is advancing through the prehaustoria towards the surface of the host (see arrow) rather like a lateral root emerging from the pericycle.

In some cases shoot tips formed tight coils which did not encompass a petiole. These coiled regions developed prehaustoria which were morphologically and structurally different to those formed on tight coiled regions which surrounded a petiole. The prehaustoria on unattached coils were much more elongated and did not have lobed cells at the epidermis. The SEM micrograph in Fig I.2j shows an elongated unattached haustorium. A cross-section of one of these prehaustoria (Fig I.2k) shows the elongation of the structure without a differentiating haustorium at the centre.

This experiment showed that in attached coils the haustorium reached the interface between the host and parasite between 3 and 4 days after inoculation. The haustoria then emerged from the surface of the prehaustoria and penetrated the host. The SEM micrographs shown in Figs I.2. l and m demonstrate how the haustorium emerged from the prehaustorium and neatly punctures the surface of the host. The surface of the parasite which had been in contact with the host is unusually smooth compared to the normal parasite stem surface. It appears that some substance exuded by the parasite has formed a complete connection with all the available surface of the host.

The events post penetration

Each prehaustorium along the tight coiled region developed a haustorium and penetrated the host. The first stage of penetration involved the whole haustorium pushing into the petiole like a nail into wood (see Fig I.2n). The haustorium enters at right angles to the surface of the petiole. Immediately outside the haustorium inside the host is a deeply

Fig. I.2g. Transverse section through part of the coiled region of a *Cuscuta campestris* stem three days after inoculation onto *Pelargonium* petiole. Two prehaustoria are shown. The superficial cells of one of them have become lobed. Haustorial initials (HI) are seen in the centre of the prehaustoria. Sub-epidermal cells (SEL) Technical details : Light microscope, I.0µm thick, resin embedded, Touidine blue stained, magnification X 95 .▼

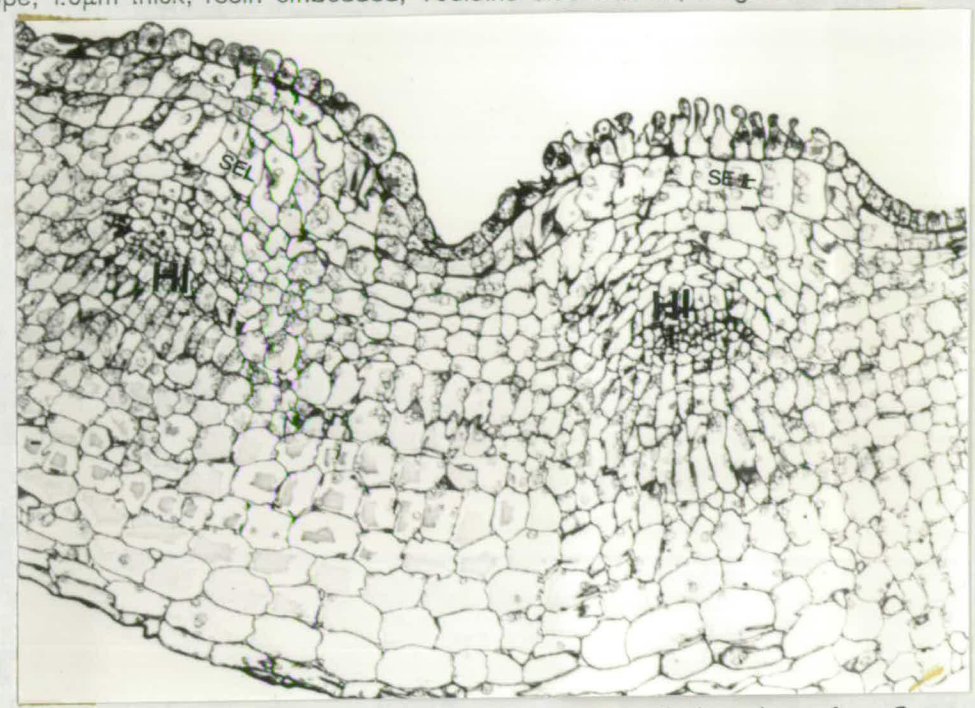


Fig. I.2h. Part of transverse section through a light coiled region of a *Cuscuta campestris* (P) shoot tip infecting a *Pelargonium* (H) petiole. The superficial cells of the prehaustorium are seen to be much lobed (LS) and perfectly moulded to the surface of the host epidermis (E). Technical details : Light microscope, 0.5µm section, resin embedded, Toluidine blue stained, magnification X 920 .▼

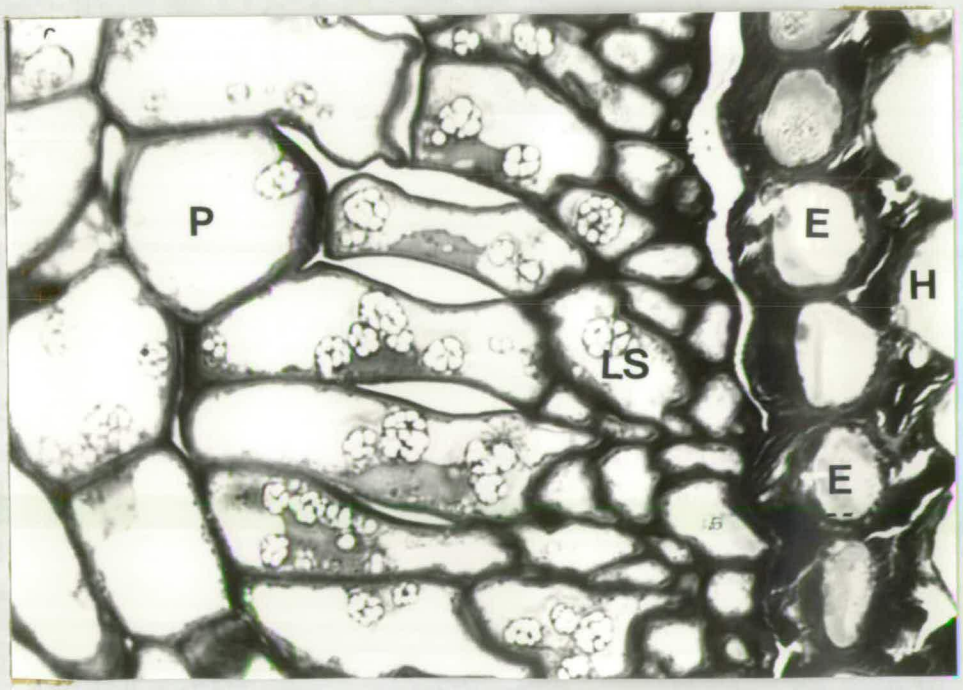


Fig. I.2i. Transverse section through part of the coiled region of a *Cuscuta campestris* (P) shoot tip infecting a *Pelargonium* (H) petiole showing a single prehaustorium containing a developing haustorium (Ha) Technical details : Light microscope, 0.5µm, resin embedded, Toluidine blue stained, magnification X 95 ▼

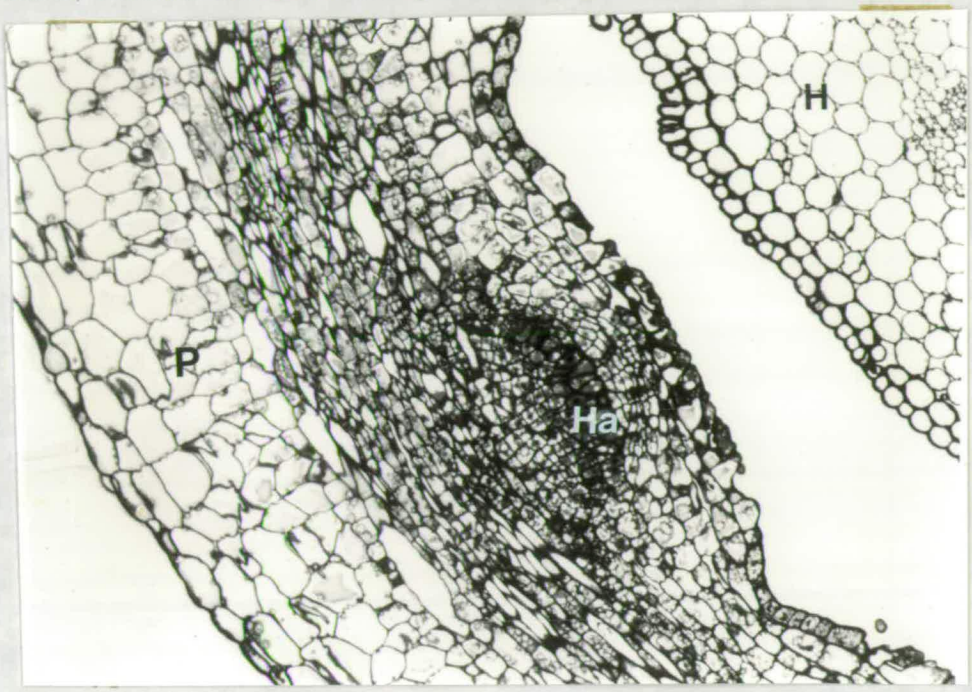


Fig. I.2j. SEM micrograph showing a single unattached prehaustorium. It is elongate relative to attached prehaustoria and no lobed cells are visible.▼

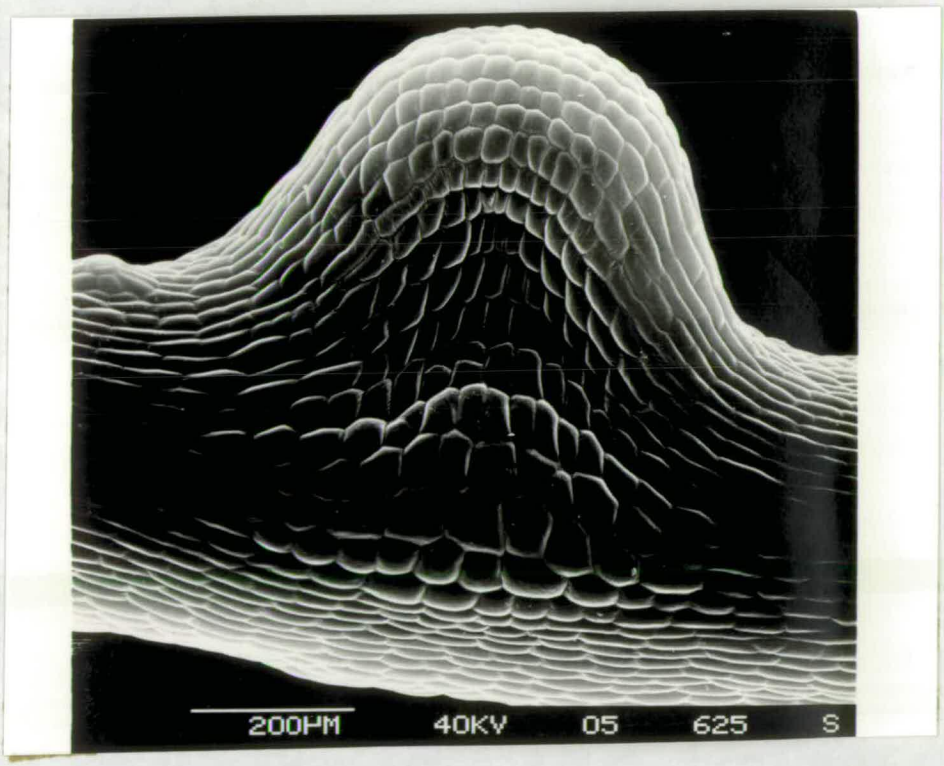


Fig. I.2k. Transverse section through a single unattached prehaustorium and stem. The prehaustorium is very elongate with no obvious differentiation of a haustorium and no lobing of superficial cells. Technical details : Light microscopy, 0.5µm thick, resin embedded, Toluidine blue stained, magnification X 95 .▼

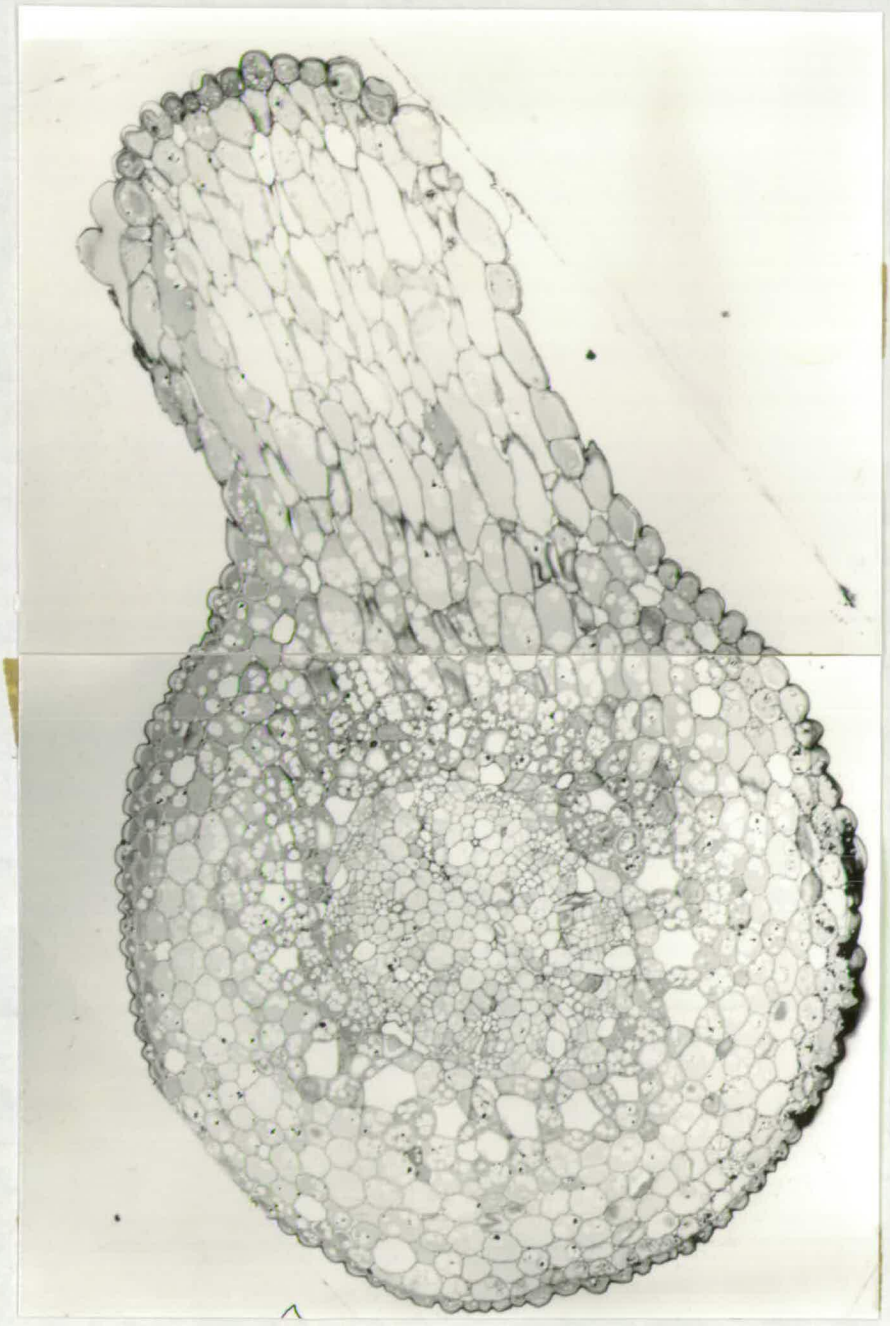


Fig. I.2l. SEM micrograph showing the smooth surface of the prehaustorium (Ph) which was in direct contact with the epidermis of a *Pelargonium* petiole. A haustorium (Ha) has emerged from the prehaustorium. Epidermal hairs from the host have punctured holes in the prehaustorium as the parasite has appressed closely to the host. ▼

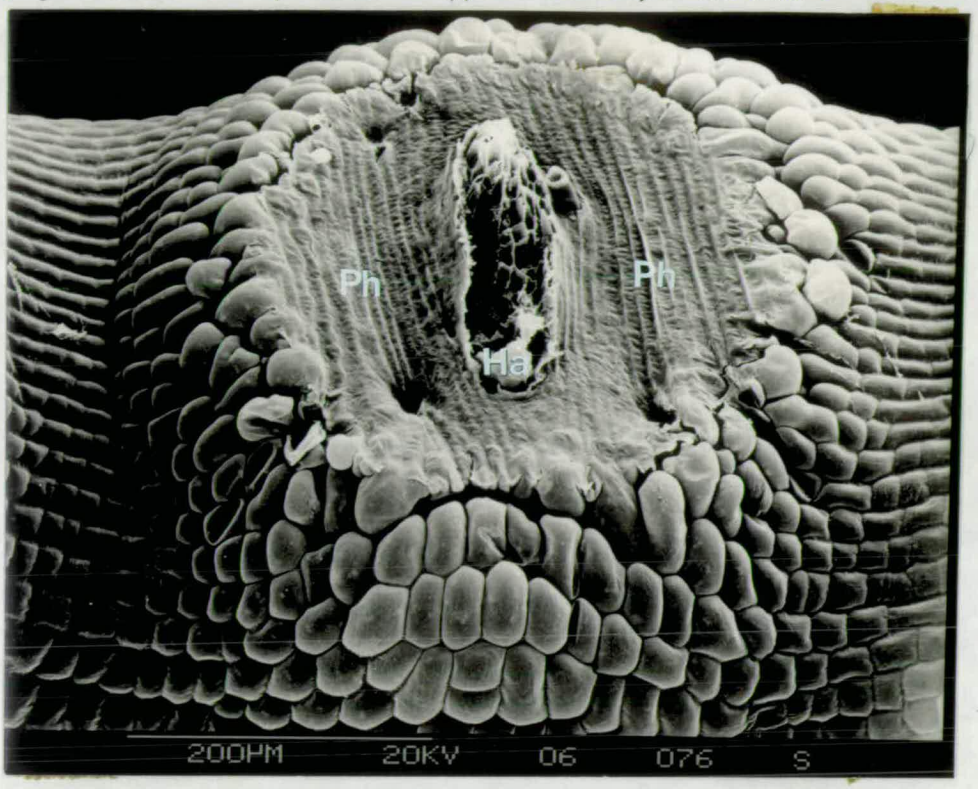


Fig. I.2m. SEM micrograph showing the surface of a *Pelargonium* petiole which has been penetrated by two *Cuscuta campestris* haustoria (Ha). The rest of the parasite has been removed. Damage adjacent to the points of penetration is minimal.▼

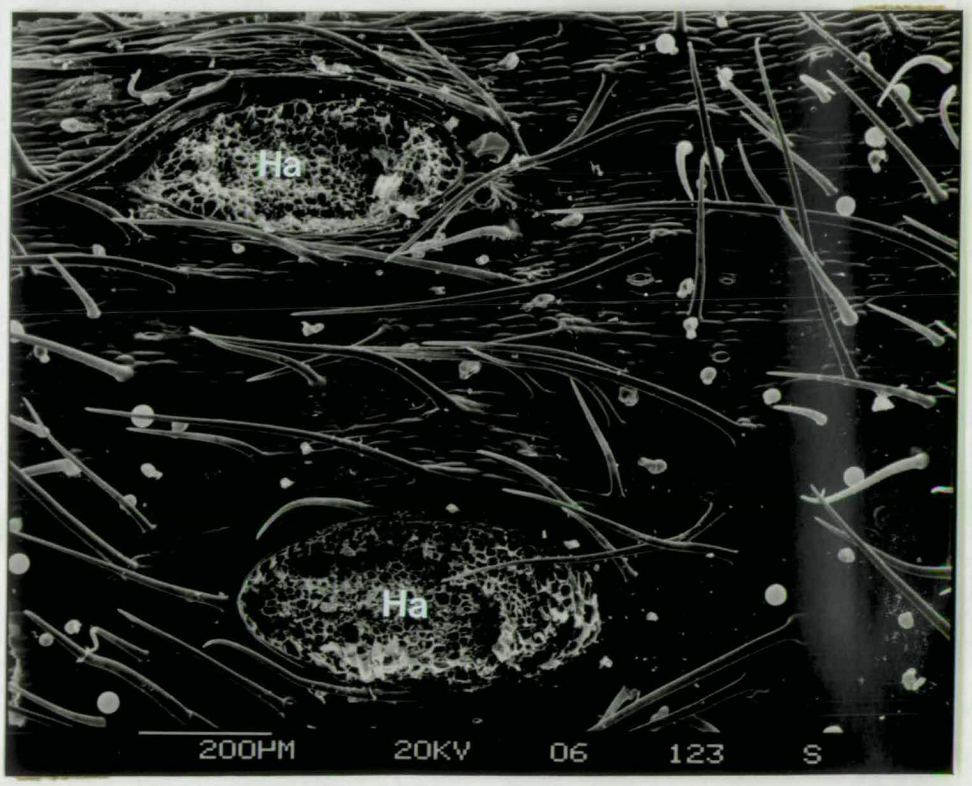
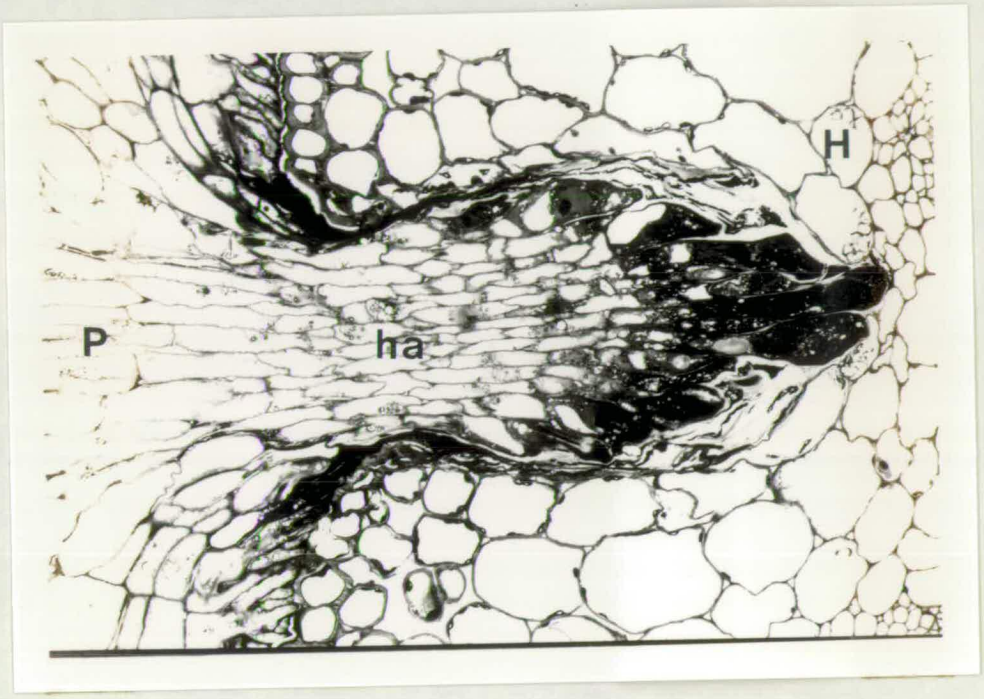


Fig 1.2n Transverse section through part of a coiled region of *Cuscuta campestris* (P) infecting a *Pelargonium* (H) petiole showing the initial penetration of a single haustorium (ha) The cell walls of the parenchymatous tissue in front of the haustorium are seen to be compressed together as the parasite pushes into the host. Technical details : Light microscopy, 0.5µm section, resin embedded, Toluidine blue stained, magnification X 225



stained layer. The cells of the host appear to have been squashed and the walls of the cells compacted against each other by the invading haustorium. The whole haustorium pushed into the host to a depth of approximately 0.2mm. Following this, the superficial cells of the haustorium began to extend independently in all directions into the host. The movement of these filaments or 'search hyphae' is illustrated in Fig.I.2o. The figure shows two haustoria. One has many search hyphae which are swollen and folded at the ends in contact with the sclerenchyma of the host as if there were some difficulty in penetrating it. The other has successfully penetrated this layer. Once through this layer, search hyphae appear to be growing towards the vascular bundle (Vb). In the centre of the haustoriumshown in Fig 1.2p secondarily thickened cells joined end to end and are visible. A continuous channel of thickened cells is not visible in the plane in which this section was taken. Apart from the cells which must have been physically crushed during the original penetration, the host cells surrounding the haustorium show no visible changes or disruptions. Even the cell which appears to be penetrated with a parasite cell (Pe) does not appear to have responded very vigorously.

Transverse sections of penetrated host petioles show how the search hyphae appear to grow both inter-(Fig I.2q) and intra-cellularly (Fig I.2r) as they travel through the host. Fig I.2q (TEM micrograph) shows how the search hyphae (sh) move along the middle lamellae between the sclerenchyma (sc) cells. Part of a *Cuscuta* cell is seen forming what appears to be a wedge (W) between host cells. The main body of the hypha (h) has developed a process out into the available space at the junction between two cells. Fig I.2r, a light micrograph, shows how search hyphae are able to travel intra-cellularly through host parenchyma. The search hyphae have broken through the sclerenchyma and are pushing into the parenchyma. The hypha at A seems to be causing the invagination of a host cell. The host cell-wall seems to be curled back at the point of entry (E) of the hyphae into the cell rather than being sharply cut or dissolved. The hypha at B also appears to have entered a host cell. The host/parasite cell wall complex at C appears to be thickened. The width of stained material is thicker than might be expected from the combined width of host and parasite cell walls.

The close contact between host and parasite became more clear in an electron microscope study of *in vitro* interactions described in section 2. The walls of filaments which have made contact with the host vacular bundles became thickened with lignin. This was shown using the metachromatic staining properties of Toluidine Blue. Fig I.2s shows the distinctive vascular tissues extending from the host vascular bundle to the base of the haustorium in the main body of the parasite. This was presumably the formation of a vascular connection between the host and the parasite. The host cells which were not actually penetrated or crushed by the parasite did not appear to be affected by its

Fig. I.2o. Transverse section through part of a coiled region of *Cuscuta campestris* infecting a *Pelargonium* petiole showing two haustoria which have penetrated the host. The initial invasive growth of the haustoria has ceased and the search hyphae are extending into the host. The search hyphae of one haustorium appear swollen and compressed against the host sclerenchyma (Sc). Those of the other have penetrated it and one growing towards the vascular bundle (Vb) (see arrow). Technical details : Light microscopy, 0.5µm, resin embedded, Toluidine blue stained, magnification X 95 ▼

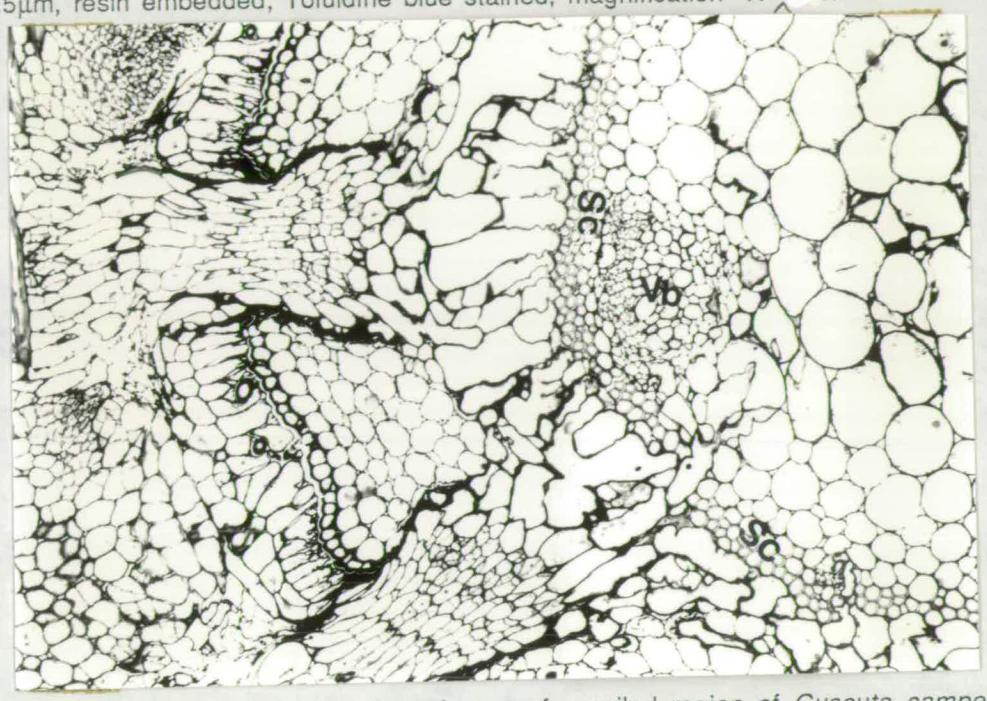


Fig. I.2p. Transverse section through part of a coiled region of *Cuscuta campestris* (P) infecting a *Pelargonium* (H) petiole showing the differentiation of lignified cells (LC) at the centre of the haustorium and adjacent to the vascular bundle (ub). Technical details : Light microscope, 0.5 µm section, resin embedded, Toluidine blue stained, magnification X 225 ▼

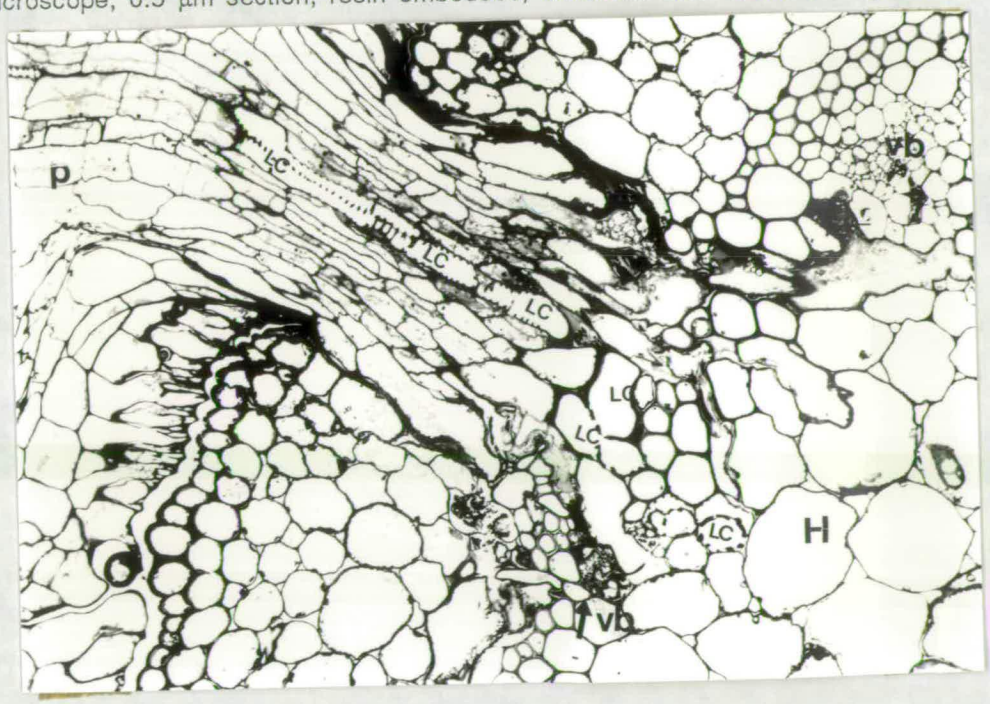


Fig. I.2q. TEM micrograph showing search hyphae (sh) of *Cuscuta campestris* (P) deforming and pushing into the spaces between individual sclerenchyma (sc) cells in *Pelargonium* petioles. Technical details : magnification =X 2000 ▼

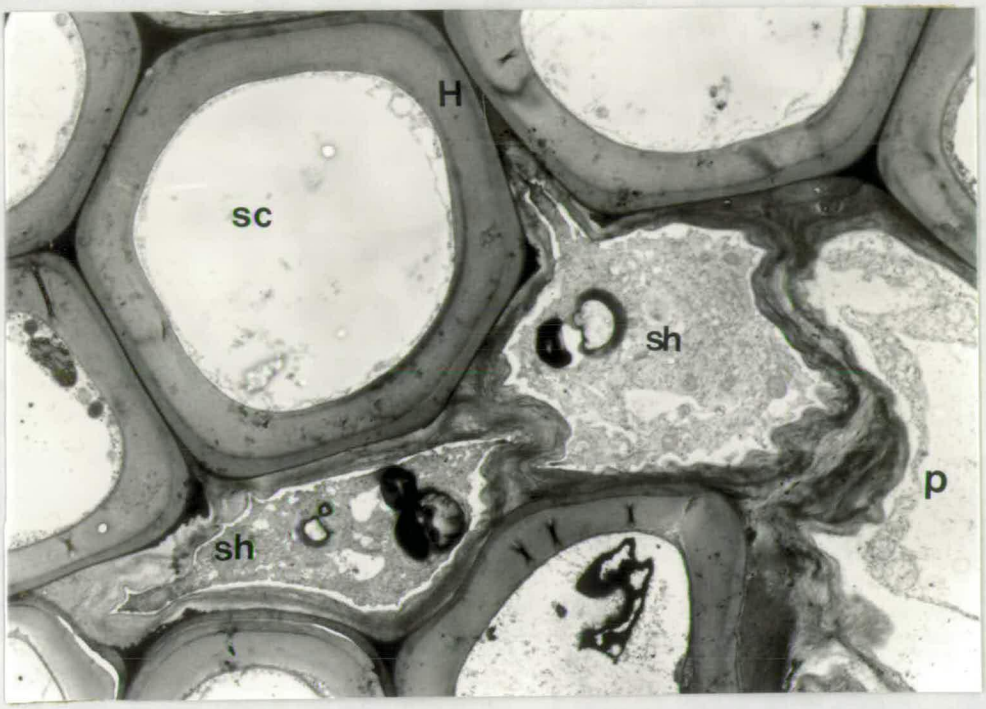


Fig. I.2r. Transverse section through part of a tight coiled region of *Cuscuta campestris* (P) infecting a *Pelargonium* (H) petiole showing the penetration of the sclerenchyma (Sc) and cortex of the host by search hyphae (sh). Search hyphae at A and B appear to have penetrated the host cells. (E) = entry point into cell (C) = host/parasite cell wall complex. Technical details : Light microscopy, 0.5µm section, resin embedded, Toluidine blue stained, magnification X 570 ▼

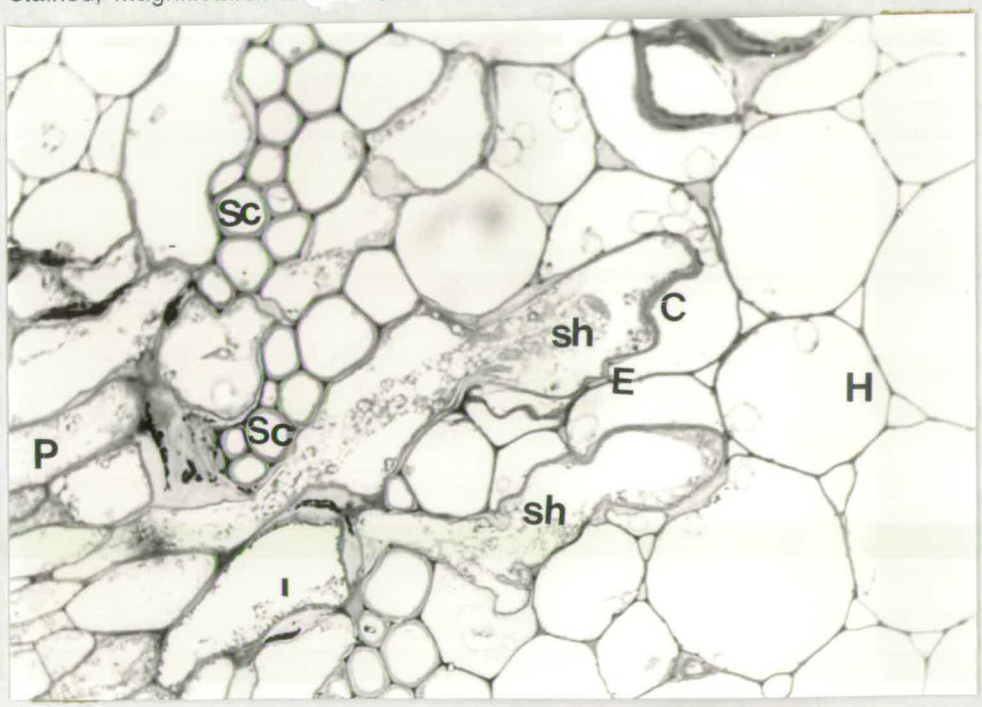
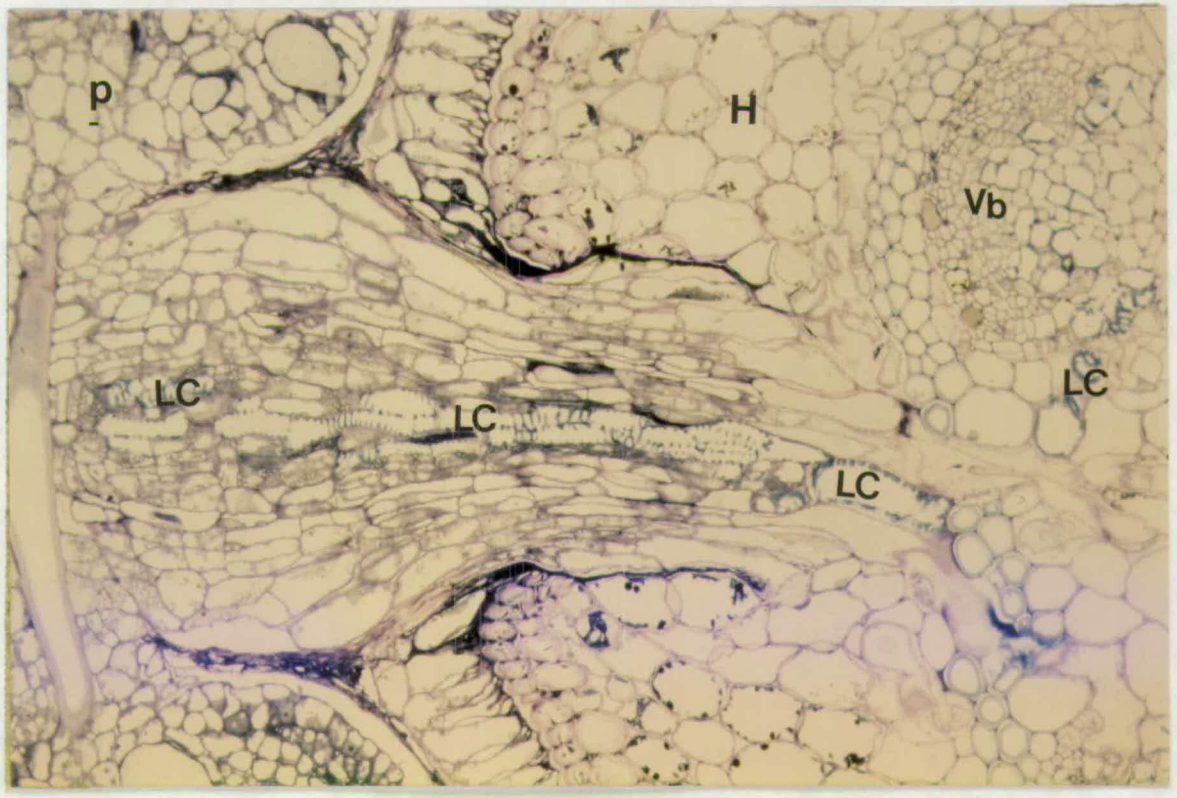


Fig. I.2s. Transverse section through part of a tight coiled region of *Cuscuta campestris* (P)infecting a *Pelargonium* (H) petiole showing the development of a lignified channel. Metachromatic staining with Toluidine blue shows the differentiation of lignified cells (LC) in the central region of the haustorium and adjacent to the vascular bundle (Vb). Technical details : Light microscopy, 0.5 µm, resin embedded, Toluidine blue stained, magnification = X 325



presence. There was no evidence of unusual patterns of division or necrosis.

The following major points arise from this experiment:

(I) The cells of uncoiled stems of *Cuscuta campestris* are often densely cyloplasmic and contain numerous starch grains.

(2) The petioles of *Pelargonium* consist of a central region containing several vascular bundles interspersed with parenchyma cells. This central region is surrounded by a complete double ring of sclerenchyma. External to this are several layers of parenchyma.

(3) The coiling of *Cuscuta campestris* involves greater longitudinal expansion of the cells on the side of the stem not in contact with the host.

(4) The development of prehaustoria involves the elongation of the epidermis and sub-epidermal layers.

(5) The parasite can develop prehaustoria whether in contact with a host or not. Prehaustoria not in contact with a host elongate greatly but do not appear to differentiate a substantial haustorium structure inside them.

(6) The prehaustorial cells which are in direct contact with a host become much lobed. The surface of the parasite becomes perfectly moulded to the surface of the host. This is achieved by the exudation of some unknown substance presumably from these lobed cells.

(7) The haustoria arise from within the prehaustoria and grow towards the interface between the host and parasite.

(8) The penetration of the host is a two-step process. This involves an initial entry of the parasite by the whole mass of the haustorium followed by the extensive ramification of individual cell filaments into and between the cells of the host.

(9) The cells of the host are pushed aside during the initial entry of the haustorium. These cells collapse to form a deeply staining cell wall complex. When search hyphae enter *Pelargonium* cells the host walls appear to invaginate rather than being punctured or completely removed by the parasite.

(I0) When search hyphae have penetrated the sclerenchyma layer and come into contact with vascular bundles of host cells, lignin begins to be deposited in the walls of the parasite cells. Adjoining cells, from the point of contact with the host vascular bundle to the base of the haustorium, become thickened. In the main body of the haustorium, thickening is confined to the central cells.

The results presented in this section have contributed to a better understanding of the events which occur when *Cuscuta campestris* invades *Pelargonium zonale* petioles. These results act as a reference point for the work in the next section. Section 2 of this chapter now follows with the description of the development of an *in vitro* system to study the host/parasite interaction.

Section 2

The development of an *in vitro* system to study the interactions between *Cuscuta campestris* and *Pelargonium zonale.*

The advantages of an *in vitro* system to study this host/parasite interaction are described in Ch I. This section is concerned with the development of such a system in which standard amounts of cultured host (*Pelargonium* petioles) and parasite tissues are brought together in a controlled sterile environment. The first step was to choose the types parasite tissues which would be used in the system.

Types of parasite tissues

The are are two forms in which *Cuscuta* can be inoculated onto host plants and result in a successful infection, as sterile germinated seedlings (see Fig 2.1.3a Ch2); or as excised shoot tips (see Fig 1.1a Ch3). Seedlings of *Cuscuta campestris* can be simultaneously sterilised and stimulated to germinate (60% germination). However, seedlings are fragile and easily damaged during inoculation. It was also found that the time between inoculation and coiling is unpredictable and often prolonged. On the other hand excised shoot tips could be easily orientated against the host and coiling usually occurred within 24hr. Another point in favour of using excised shoot tips was that all the material used could be derived from a single plant. Under these circumstances all the plant material can be said to be genetically identical. Despite *Cuscuta campestris* plants being cleistogamous, it was unlikely that individual seedlings germinated from supplied seed would be genetically identical. As it was desirable to reduce the amount of variation in the system, shoot tips were chosen to be the infecting agent in the *in vitro* system.

As described in Ch 1 the sterile *Cuscuta* inocula were to be inoculated onto cultured *Pelargonium* petioles.

Standardisation in the system

Strenuous attempts were made to standardise the interaction between the host and parasite. This meant bringing together standard amounts of host and parasite in a precise manner, under controlled conditions. Standard lengths of shoot tips were to be inoculated on to the host in a standard repeatable manner. Each shoot tip had to be capable of coiling and progressing towards a successful infection. Little was known about how the ability of excised shoot tips to infect was related to their length. Neither was it known how excision affected the shoot tip nor how rapidly the ability to infect a host decreased with

time. To repair this lack of knowledge a series of experiments was performed to investigate the properties of excised shoot tips, paying particular attention to factors which affected their ability to infect. These experiments are described below.

2.I Properties of Excised *Cuscuta campestris* stem tips

Once germinated the shoot apex of *Cuscuta* seedlings continue to grow in length until a host is found or food reserves are exhausted. An experiment was performed to see if shoot tips were able to mobilise their food reserves in a similar way when excised from the mother plant.

2.I.I The Elongation of *Cuscuta campestris* stem tips following excision from the rest of the plant.

The aim of this experiment was to see if excised shoot tips were able to mobilise their food reserves and extend when excised from the rest of the plant.

Twenty-one 30mm and twenty 40mm shoot apices were excised with a sharp razor blade from clonal *Cuscuta campestris* plants grown in the greenhouse. Two sizes of shoot tip were chosen in order to see if the response was different for shoot tips of different lengths. The cut end of each tip was immersed in distilled water inside a Durham tube. The tubes were placed in racks inside a growth cabinet with constant fluorescent illumination of 10-15 μmoles $m^{-2}s^{-1}$ and a temperature of 25^{o}C. After 32 hours the lengths of ten 30mm inocula and ten 40mm inocula were measured. The lengths of all the shoot tips were measured after 48 and 94hr. The means and standard errors of the lengths of each type of tip at each time interval were calculated.

The Change in the mean length (Fig 2.I.Ia and Table 2.I.Ib)

Excised shoot tips whether 30 or 40mm, increased in length between all the times measurements were taken up to 94hr. Table 2.I.Ic shows the mean rate of extension of the shoot tips between the times when measurements were taken. The mean rate of extension of the 30mm shoot tips decreased with each subsequent measurement. That for 40mm tips increased between 32 and 48hr before decreasing again between 48 and 94hr. The mean rate of extension of 40mm shoot tips was greater than those for 30mm shoot tips over the same time periods (0-32hr, 32-48hr, 48-94hr). This experiment demonstrated that shoot tips extend when excised from the rest of the plant, when supplied with a source of water. Excised shoot tips were able to mobilise their energy reserves and extend in search of a host.

Fig. 2.1.1a. The change in the mean length of excised 30 and 40mm *Cuscuta campestris* shoot tips with time when their cut ends were immersed in water. (I) = standard error bars.

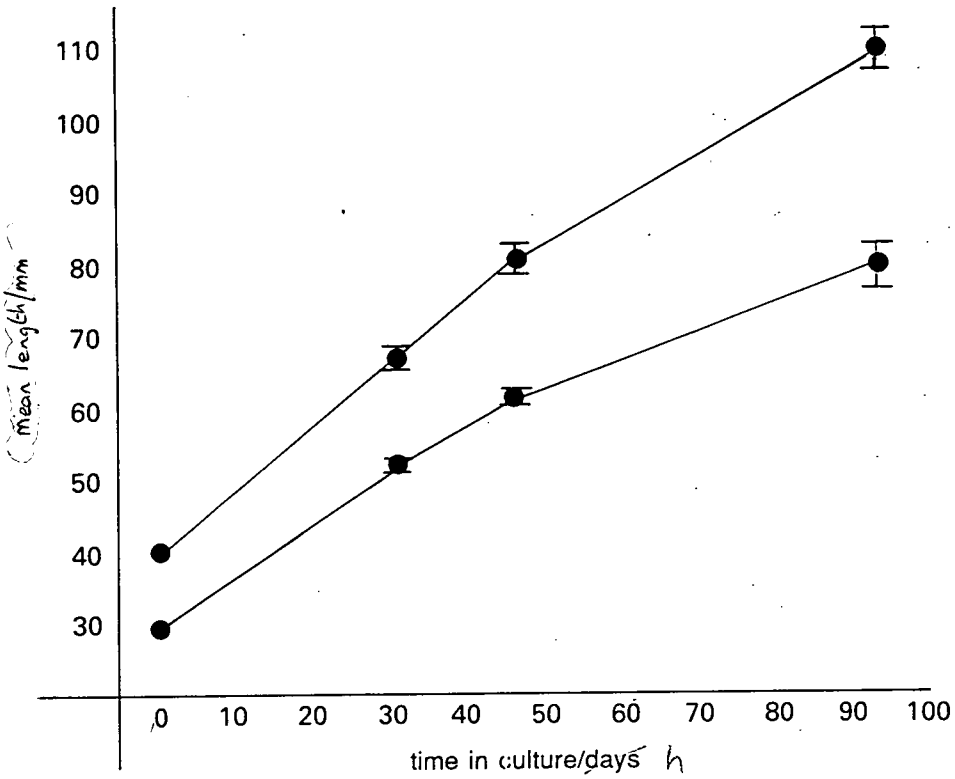


Table 2.1.1b. The length of excised *Cuscuta campestris* shoot tips (originally 30 and 40mm) after 32, 48 and 92 hr incubated in distilled water. Lengths shown are in mm.

tips originally 30mm			tips originally 40mm		
32hr	48hr	94hr	32hr	48hr	94hr
48	59	91	61	87	103
56	57	86	64	76	98
50	58	78	74	91	120
56	69	76	66	85	113
49	47	51	77	80	120
54	54	67	65	87	121
53	63	67	66	86	95
51	65	68	73	80	115
51	64	84	65	83	139
52	68	102	57	81	130
	65	94	70	90	118
	62	75		78	97
	59	69		86	101
	57	88		64	120
	69	101		78	120
	62	80		92	95
	68	85		78	111
	64	83		86	76
	67	66		70	104
	57			66	
	61				

Table 2.I.1c The mean rate of extension *Cuscuta campestris* shoot tips (mm hr^{-1}) which were excised from the rest of the plant and incubated in water.

Size at excision (mm)	Mean rate of extension / mm hr^{-1}		
	0-32 hr	32-48 hr	48-94 hr
30	0.687	0.594	0.392
40	0.847	0.882	0.633

The optimum shoot tip length

Having confirmed that excised shoot tips behave in a similar manner to the shoot system of germinated seedlings it was necessary to determine the most effective length of inoculum which would ensure successful infection of the host. In order to standardise the inoculation procedure it was necessary to decide on a fixed length of shoot tip. A short length of stem was easier to manipulate than a long piece. However, each tip which was inoculated must contain enough reserves to enable coiling and penetration of the host. An experiment was performed to determine how the ability to coil was related to length.

2.I.2 The relationship between the ability to coil and successfully infect the host and inocula length

The aim of this experiment was to see how the ability of the shoot tip to infect the host was related to the length of the inocula.

Experimental details

In this preliminary experiment excised shoot tips I0, 20, 30, 40 and 50mm long were inoculated on to petioles of greenhouse-grown *Pelargonium* plants. Eight to ten replicates of each length of shoot tip were inoculated in the manner described in section I of Ch2. After 5 days the number of shoot tips which had coiled around the hosts was recorded. Some shoot tips formed tight coils but not around the host. These coils resembled brightly coloured springs. After I2 days the number of coils which were still alive was recorded.

The frequency of coiling (Fig 2.I.2a)

None of the I0mm inocula formed tight coils. Twenty percent of the 20mm inocula coiled, but only I0% around the host. When the length was increased to 30mm 60% of the shoot tips coiled. However, still only I0% coiled around the host. All the 40mm inocula responded to inoculation by coiling and 80% of these coiled around the host. Only 90% of the 50mm shoot tips coiled and only 50% around a host petiole.

Coils still alive after I2 days (Fig 2.I.2b)

Not all the shoot tips which coiled around the host were still alive after I2 days. The coils formed around the host by 20 and 30mm inocula were still alive. However, three of the coils formed by the 40mm inocula and one formed by a 50mm inoculum had dried out by I2 days.

This experiment shows that the coiling frequency of inoculated shoot tips increases with increasing inocula length. However, coiling was not always followed by successful infection. Some shoot tips became dry and shrivelled after they had reached the coiling

Fig. 2.I.2a. The proportions of shoot tips of different lengths which had (a) coiled and (b) coiled around a host 5 days after inoculation. ☐ = shoot tips coiling

▨ = shoot tips coiling around the host

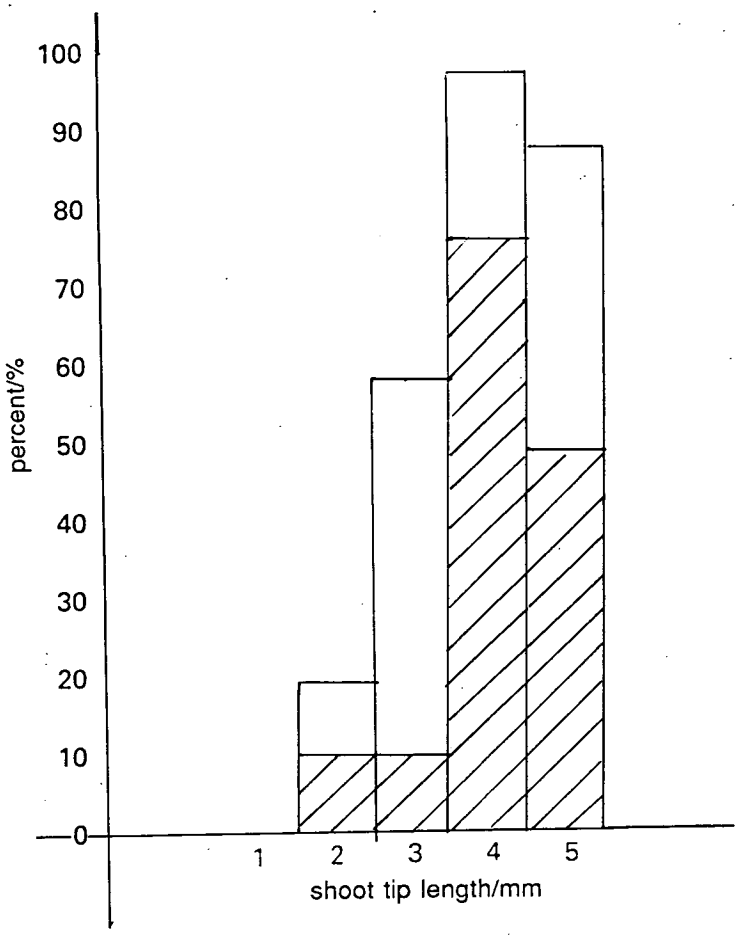
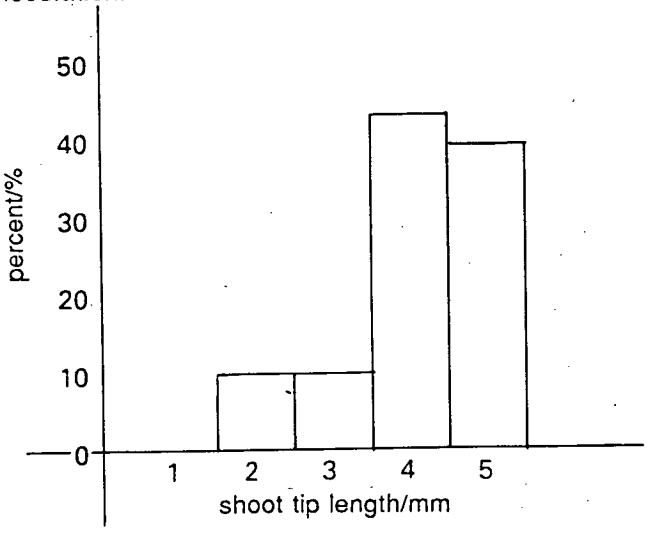


Fig. 2.I.2.b. The proportion of shoot tips of different lengths still alive twelve days after inoculation.



stage. Following this experiment it was decided to perform a more detailed experiment in order to monitor more closely the progress of inocula of different length through the successive stages of infection. In addition it was decided to determine how long the shoot tips retained the ability to infect once excised and stored in water.

2.I.3. The effect of the inoculum length and a delay in inoculation following excision, on the ability of excised shoot tips to infect the host

The aim of this experiment was to see if the ability of the parasite to coil and successfully infect the host changed with length of inoculum and with storage in water for 24 and 48 hr following excision but prior to inoculation.

Experimental

Ten 30mm, nine 40mm, ten 50mm and ten 60mm shoot tips were excised with a sharp razor blade from clonal *Cuscuta campestris* vines grown in the greenhouse. Their cut ends were immersed in distilled water in Durham tubes. They were then placed under greenhouse conditions (see section I of chapter 2). After 24hr a further eight 30mm, nine 40mm, ten 50mm and ten 60mm tips were treated in the same way. Following a further 24hr ten 30mm, nine 40mm, ten 50mm and six 60mm shoot tips were excised. These freshly excised tips along with those which had been stored in water for 48 and 24 hours were inoculated on to petioles of greenhouse-grown *Pelargonium* plants as described in section I.2 of Ch2. Several petioles on an individual plant were inoculated with parasite shoot tips. Each day the shoot tips were observed. The following was noted for each tip:

(I) Whether coiling had occurred.
(2) Whether prehaustoria had developed.
(3) Whether stomatal appendages had developed.
(4) Whether tip advance had occurred.
(5) The length of the advancing tip.
(6) Whether there was any visible host response such as discolouration or swelling.

After I4 days all the coils which were still alive were harvested and the length of their concave edges measured. Infections were considered 'successful' if tip advance had been achieved.

The effect of the length on the ability to infect

The results for freshly excised shoot tips of different lengths are described first.

The proportions of inocula of each length achieving each stage of infection (Table 2.I.3a)

All the 50mm and 60mm inocula formed tight coils around the petioles onto which they were inoculated. Shorter 30 and 40mm inocula coiled around their hosts in only 60 and 80% of inoculations respectively. Only 50% of 30mm inocula went on to develop prehaustoria all of which dried out before developing papillate appendages. None of the 30mm inocula had successful infections. Seventy seven percent of the 40mm inocula reached the prehaustorial stage, 80% the papillate appendage stage and 70% tip advance. All the 60mm inocula completed all of the morphological stages. The longer the inocula, the more morphological stages of infection were achieved.

The mean number of days taken by inocula of different lengths to reach each stage of infection (Table 2.I.3b)

The mean number of days inocula took to reach tip advance decreased with increasing length between 40 and 60mm. The same was true for the mean time taken to develop papillate appendages. These differences are not however statistically significant.

The mean length of coiled regions for inocula of different lengths (Table 2.I.3c)

The length of the coil region or 'coil length' is the total length of the concave side of the tight-coiled region made around the host during infection. Measurements were taken I4 days after inoculation. The number of coiled regions formed and remaining after I4 days decreased with decreasing inoculum length. From the data available the mean coil length was found to increase with increasing length for 30, 40 and 50mm inocula. No measurements were made for 30mm inocula because the coils which had formed dried up by I0 days after inoculation.

This part of the experiment shows

(I) That as the inoculum length is decreased below 60mm the number of morphological stages of infection that a shoot tip is likely to achieve decreases.

(2) The mean coil length increases with increased inocula length between 40 and 60mm.

The effect of delayed inoculation

The results for the freshly excised tips are now compared to those for shoot tips stored in water for 24 or 48hr before being inoculated.

The proportion of inocula of each length and treatment which achieved each stage of infection (Table 2.I.3a)

Table 2.1.3a The percentage of *Cuscuta campestris* shoot tip of given length which achieve a given stage of infection when inoculated onto *Pelargonium* petioles.
PH = prehastoria, PA = papillate appendages, TA = tip advance and HS = host swelling.

Tip length (mm)	Time in water (hr)	number of replicates	percentage coiling	percentage forming PH	percentage forming PA	percentage reaching TA stage	percentage causing HS
30	0	10	60	50	0	0	0
30	24	8	100	100	50	50	0
30	48	10	100	80	50	50	40
40	0	9	89	77	22	11	0
40	24	9	100	100	66	55.5	11
40	48	7	100	100	57	57	20
50	0	10	100	100	80	70	30
50	24	10	100	90	90	90	20
50	48	8	100	100	100	100	75
60	0	6	100	100	100	100	33
60	24	9	100	100	55.5	55.5	25
60	48	10	100	100	80	80	10

Table 2.I.3b The mean number of days taken for *Cuscuta* shoot tips to achieve a given stage of infection when inoculated onto *Pelargonium* petioles.

Tip length (mm)	Time in water (hr)	Number replicates	Time taken to develop PA (days)	Standard error SE	Time taken to reach TA (days)	standard error SE
30	0	10	-	-	-	-
30	24	8	6.0	0	6.0	-
30	48	10	5.6	± 0.479	6.8	± 0.546
40	0	9	5.5	± 0.236	8.0	± 0.252
40	24	9	6.0	-	6.4	± 0.516
40	48	7	6.2	± 0.621	6.5	± 0.378
50	0	10	5.12	± 0.112	6.28	± 0.239
50	24	10	6.10	± 0.368	6.44	± 0.279
50	48	8	6.22	± 0.493	6.25	± 0.249
60	0	6	4.83	± 0.167	6.0	0
60	24	9	7.2	± 0.365	8	-
60	48	10	5.5	± 0.321	6.5	± 0.292

Table 2.1.3c. The mean length of the coiled regions made by the parasite around the host by shoot tips of different length incubated for different times in distilled water prior to inoculation.

Time in water (hr)	original size of tip (mm)	number of replicates	mean coil length (mm)	standard error s.e.
0	30	-	-	-
0	40	2	19.50	± 1.50
0	50	7	25.80	± 1.09
0	60	6	33.00	± 2.16
24	30	7	14.10	± 2.26
24	40	8	23.75	± 2.12
24	50	9	28.33	± 2.13
24	60	5	26.20	± 4.62
48	30	5	24.40	± 1.63
48	40	7	28.60	± 1.57
48	50	8	35.10	± 1.39
48	60	10	25.10	± 5.58

Table 2.I.3d The distribution of different types of swelling shown by *Pelargonium* petioles infected with *Cuscuta campestris* shoot tips.

Length (shoot tips mm)	Time in Water (hr)	percentage of shoot tips	
		+ or -	+
30	0	-	-
	24	-	-
	48	0	40
40	0	-	-
	24	0	11
	48	10	10
50	0	0	10
	24	10	10
	48	37.5	37.5
60	0	0	33
	24	22	0
	48	10	0

The development of the 30mm inocula

60% of freshly excised 30mm shoot tips inoculated on to host petioles coiled around them. All the 30mm shoot tips which were first stored in water for 24 or 48hr before inoculation subsequently coiled around the host. The number of freshly excised 30mm shoot tips which went on to develop prehaustoria was only 50%, while l00 and 80% of shoot tips first stored in water for 24 and 48hr respectively went on to develop them. None of the freshly excised 30mm shoot tips went on to achieve any further stages of infection. However 50% of 30mm shoot tips first stored in water for 24 and 48hr went on to develop papillate appendages and reach the tip advance stage.

Development of the 60mm inocula

When the length of freshly excised shoot tips was increased to 40mm, 88% of them coiled around the host. All 40mm shoot tips which were first stored in water for 24 or 48 hr went on to coil when inoculated on to host petioles. Seventy-seven percent of freshly excised 40mm shoot tips developed prehaustoria. All those stored in water prior to inoculation developed these organs. Only 22% of the freshly excised 40mm shoot tips went on to develop papillate appendages. Sixty six per cent of shoot tips stored for 24hr in water developed papillate appendages and 57% of those stored in water for 48hr did so. Finally, l0% of freshly excised 40mm shoot tips reached the tip advance stage while those stored in water for 24 and 48hr reached this stage in 55.5 and 57% of cases respectively.

The development of the 50 and 60mm inocula

For 50 and 60mm shoot tips there were no differences in the proportions of replicates which achieved either coiling or developed prehaustoria for freshly excised tips or those stored in water. All the inoculated shoot tips reached these stages. However there were some differences in the proportions of shoot tips which developed papillate appendages. While 80% of freshly excised 50mm shoot tips developed papillate appendages, 90 and l00% of those stored in water for 24 and 48hr respectively developed them.

All the 50mm shoot tips which were stored in water for 24 or 48hr and which developed papillate appendages also reached the tip advance stage. Only 70% of freshly excised 50mm shoot tips reached the tip advance stage. Freshly excised 60mm shoot tips all achieved all stages of infection. Not all 60mm shoot tips which were first stored in water before inoculation completed all stages: only 55.5% of those stored in water for 24hr developed papillate appendages and reached the tip advance stage; eighty percent of 60mm inocula which were stored in water for 48hr developed papillate appendages and reached the tip advance stage. All the tips which were stored in water increased in length before they were inoculated.

The time taken to infect the host

The mean number of days that each group of tips took to complete each stage of infection are presented in Table 2.I.3b The data for prehaustorial development were not obtained.

The development of papillate appendages

The 30mm inocula

None of the freshly excised 30mm inocula progressed to the stage where papillate appendages are produced. Those 30mm inocula which were stored in water for 24hr before inoculation developed papillate appendages after an average of six days. The 30mm inocula stored for 48 hr in water developed papillate appendage on average 5.6 days after inoculation.

The 40mm inocula

Freshly excised 40mm shoot tips which coiled around their host developed after an average of 5.5 days post inoculation. Those stored in water for 24 and 48hr before inoculation developed papillate appendages after an average of 6 and 6.2 days respectively.

The 50mm inocula

When the length of freshly excised inocula was increased to 50mm the time taken for inoculated shoot tips to develop papillate appendages was only 5.I2 days. Those stored in water for 24 and 48hr, then inoculated, developed papillate appendages after an average of 6.I and 6.2 days respectively.

The 60mm inocula

On a further increase in size of the inocula there was a further fall in the average number of days taken to reach the papillate appendage stage (5.I2-4.83). Those 60mm shoot tips which had been stored in water for 24 and 48 hr prior to inoculation took on average 7.2 and 5.5 days respectively to reach this stage.

The storage of 40, 50 and 60mm shoot tips in water for 24 or 48hr appeared to increase the time taken for them to reach the papillate stage relative to those not stored in water.

The extension of the shoot apex

There were also differences between stored and unstored shoot tips with respect to the time taken to reach the tip advance stage.

The 30mm inocula. None of the freshly excised 30mm inocula proceeded to tip advance. However some of the 30mm inocula which were first stored in water for 24 and 48 hr reached the tip advance stage after an average of 6.0 and 6.8 days respectively.

The 40mm inocula. Freshly excised 40mm inocula reached the tip advance stage on average 8 days after inoculation. When stored in water for 24 and 48hr inocula reached this stage on average 6.4 and 6.5 days after inoculation respectively.

The 50mm inocula. Freshly excised 50mm inocula reached the tip advance stage on average 6.28 days after inoculation. Those stored in water for 24 and 48hr took an average of 6.44 and 6.25 days to reach this stage.

The 60mm inocula. The shortest mean time to reach tip advance was shown by freshly excised 60mm shoot tips (6.0 days). However when inocula of this length were stored in water for 24 and 48hr before inoculation the average times taken were 8 and 6.5 days respectively.

The mean number of days elapsing between inoculation and tip advance was seen to decrease with the increase in the inoculum length . An opposite trend was observed for inocula stored in water for 24hr before inoculation. No trend was observed for shoot tips stored for 48hr before inoculation. None of these differences were found to be statistically significant.

The length of the coiling region for inocula of different lengths and treatments (Table 2.I.3c)

Freshly excised inocula Only 40, 50 and 60mm freshly excised inocula had coiled regions after I4 days. The mean coil length increased with the increase in inocula length. The coil lengths of 40 and 50mm inocula were not significantly different from each other at the 5% level. Neither were the coil lengths of 50 and 60mm inocula. However the mean coil lengths for 40 and 60mm inocula were significantly different at the 5% level.

Inocula stored for 24hr in water. The mean coil lengths for 30, 40 and 50mm shoot tips stored in water for 24hr before being inoculated, increased with increase in length. The mean coil length of the 30mm inocula was significantly different from those for the 40 and 50mm inocula at the 5% level. Those for 40 and 50mm inocula were not significantly different at this level. The 60mm inocula had a mean coil length smaller than that for 50mm inocula. This difference was not significant at the 5% level.

Inocula stored for 48hr in water. The mean coil length for 30, 40 and 50mm inocula increased with increase in length. When the inocula size was increased to 60mm the mean coil length decreased. The mean coil lengths for the 30-60mm inocula were not

significantly different from one another.

In some cases the coil lengths of Inocula of the same length receiving different treatments were significantly different from each other. There were significant differences at the 5% level between the coil lengths made by 50mm inocula stored in water for 48hr and those stored in water for 24hr or freshly excised. The mean coil length of 30mm inocula stored for 48hr in water was significantly larger than that for the 30mm inocula which were stored for 24hr in water before inoculation. There were no significant differences in the mean coil lengths of 40 and 60mm inocula regardless of treatment.

The swelling of petioles parasitised by *Cuscuta*

Several petioles parasitised by *Cuscuta* shoot tips swelled without discolouration directly under the tight coiled regions. There was no obvious pattern to its occurrence. The swelling did not correlate with shoot tip length or length of storage in water. It only occurred in the host-plant when the parasite had reached the papillate appendage and tip advance stages. At these stages the host had been penetrated by the parasite. The proportion of shoot tips which caused host petioles to swell under the coiled region are shown in Table 2.I.3d. None of the freshly excised shoot tips which were 30mm long produced any host swelling following inoculation. The same was true for 30mm tips which had been stored in water for 24hr before inoculation. However, 40% of the 30mm shoot tips which had been stored in water for 48 hr before inoculation had host petioles which swelled under the coils. The host swelled in ll% cases with freshly excised 40mm inocula. The same was true for 40mm inocula stored in water for 24hr. Twenty percent of the hosts of 40mm inocula which were stored in water for 48hr swelled. The host swelled in 30% of cases with freshly excised 50mm inocula. The hosts of those stored in water for 24 and 48hr were found to swell in 20 and 75% of cases respectively. When the inocula length was increased to 60mm the proportions which had swollen hosts were 33, 25 and l0% for shoot tips freshly excised, stored for 24hr and 48hr respectively. Different degrees of swelling were observed. If the swelling was indistinct it was recorded as + - and if pronounced it was recorded as +. The types of swelling found are shown in Table 2.3.3d. For the 30mm inocula stored in water for 48hr all the swellings on the host were pronounced. The same was true for the hosts of 40mm inocula stored in water for 24hr and those of 50 and 60mm which were freshly excised. The swelling which occurred in 50% of the hosts of 40 and 50mm inocula which had been stored in water for 48hr before inoculation was pronounced. The same was true for the hosts of the 50mm inocula stored in water for 24hr before inoculation. The swelling which occurred in some of the hosts of 60mm inocula which were stored in water for 24 or 48hr before inoculation was present but indistinct.

The following points arose from this experiment:

(I) That the ability to coil and successfully infect decreases with the decrease in the length of freshly excised shoot tips in the range of 60 to 30mm.

(2) The mean number of days taken to reach each stage of infection decreased with increasing inocula length between 40 and 60mm for freshly excised shoot tips. These differences were not however statistically significant.

(3) The ability of excised shoot tips to coil was retained for at least 48hr when stored in water.

(4) The probability that 30 and 40mm shoot tips would infect successfully was increased by storage in water for 24 or 48hr.

(5) In spite of having no external nutrient source, storing 30-50mm inocula in water for up to 48hr retained or improved their ability to infect.

(6) The mean coil length made by freshly excised inocula significantly increased with increasing length between 40 and 50mm. Storage in water for 48hr significantly increased the mean coil length produced by 30 and 50mm inocula.

(7) Petioles swelled in the region directly under the tight coils. Its presence or absence did not correlate with the time the shoot tips were stored in water or the inocula length. The swelling only occurred when the parasite reached the later stages of infection.

Following the experiment it was decided that all the shoot tips inoculated on to the *in vitro* host would be 50mm. Tips of this length can be easily orientated and in the majority of cases inoculation leads to successful infection of a host.

2.2

Cultured parasite

The above experiment showed it was unnecessary to supplement 50mm inocula with nutrient medium in order to ensure that infection would take place. However, it was decided that inoculating a cultured parasite on to a host would be valuable in providing a measure of chemical control. For example the parasite could be radioactively labelled via the growth medium. Therefore it was decided to inoculate the host with cultured parasite.

If the parasite was to be labelled or influenced by the culture medium it would have to be able to assimilate it. *Cuscuta* shoot tips have been found to extend when excised from the rest of the plant. It would be easy to assume that shoot tips which extended when placed in nutrient medium were assimilating it. This is not necessarily the case. An experiment was performed to confirm that excised shoot tips could be cultured and that they were able to assimilate the nutrient medium supplied to them.

2.2.1 Culture of excised *Cuscuta campestris* shoot tips

The aim of this experiment was to measure the change in fresh weight, dry weight and length of 30mm shoot tips cultured for 14 days in liquid nutrient medium in order to confirm that excised shoot tips could assimilate it.

Experimental

Fifty millimetre shoot tips were excised with a razor blade from clonal greenhouse grown *Cuscuta* vines. Following sterilisation (see section 3.3. of Ch2) each shoot tip was trimmed to 30mm with a scalpel and then cultured by the method described in section 3.6 of Ch2 in one of the following:

(1) Distilled water.

(2) Basic Medium (BM), a modified Whites medium described in section 3.6 of Ch2 with no added growth substances.

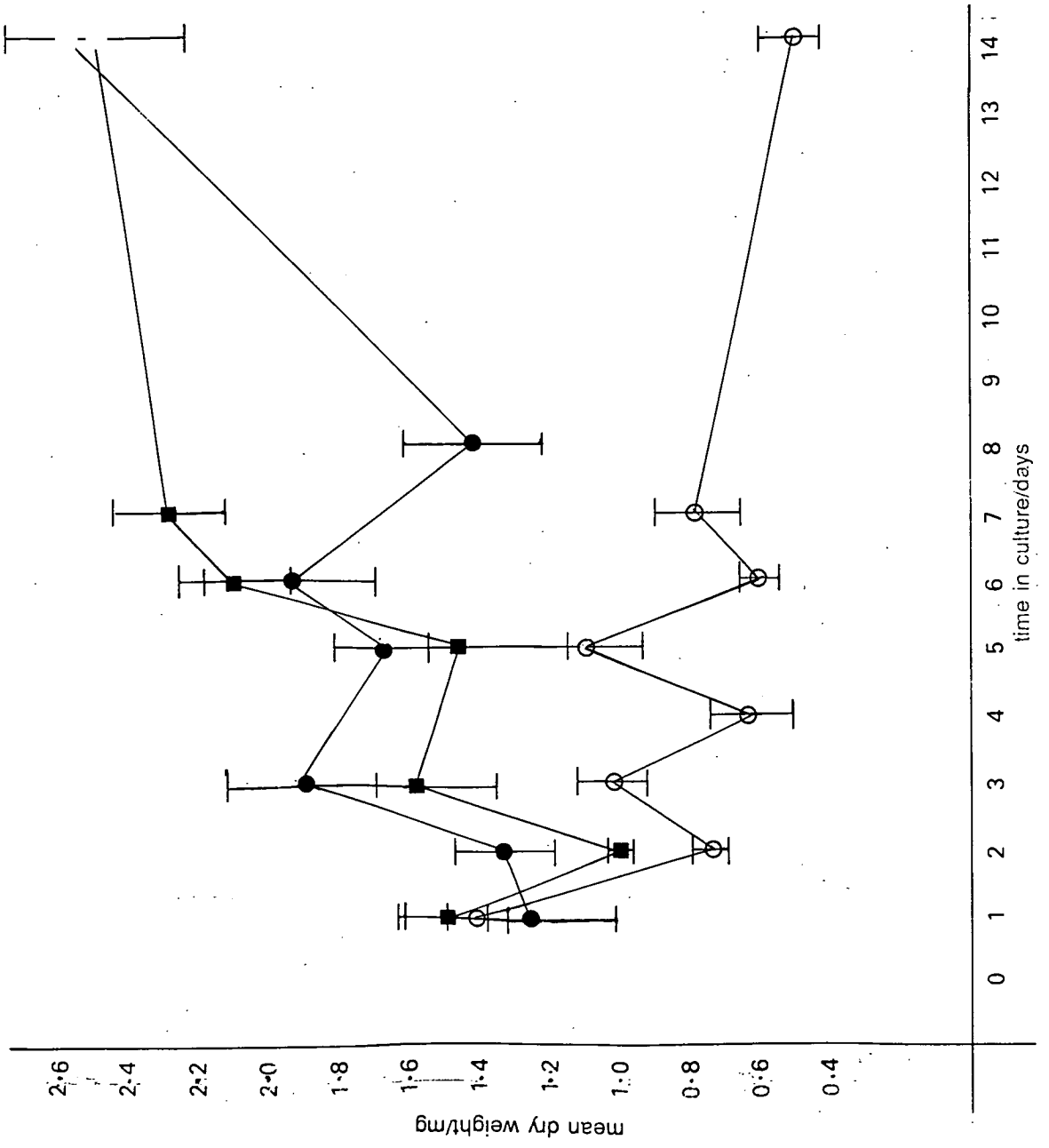
(3) BM with added Gibberelin (GA_3) to a final concentration of $1.10^{-4}M\ l^{-1}$.

Each shoot tip was numbered and then incubated in a growth cabinet under constant fluorescent illumination (light intensity 10-15 μmoles $m^{-2}s^{-1}$). The temperature was maintained at 24°C. Shoot tips grown in different media were mixed together at random along the shelf. After 1, 2, 3, 4, 5, 6, 7, 8 and 14 days samples of four tips from each treatment were harvested. Individual shoot tips were chosen at random using a table of random numbers. The fresh weight and the length in mm was measured immediately. The dry weight of each tip was measured after 48hr in a 100°C oven. The fresh and dry weights and the lengths of 10 freshly excised shoot tips were also measured. The mean, standard deviation and standard error were calculated for each sample. The mean values of length, fresh weight and dry weight for shoot tips cultured for the same period but receiving different treatments were compared using a 't' test for significance. The changes in mean fresh and dry weight and length of cultured shoot tips with time in culture are shown in Figs 2.2.1a-c.

Change in dry weight (Fig 2.2.1a)

The mean dry weight of shoot tips incubated in water decreased with time. The mean dry weight decreased between days 1 and 2. This was followed by a rise between days 2 and 3 before another fall between days 3 and 4. The mean dry weight rose again on day 5 then fell between days 5 and 6. There was a small rise on day 7 and then a decrease between day 7 and 14. The mean dry weight of shoot tips cultured in BM and BMGA increased with time in culture. For shoot tips cultured in BM the mean dry weight increased between days 1 and 2 and 2 and 3. The mean then fell slightly between days 3 and 5 before another increase between days 5 and 6. The mean dry weight decreased quite

Fig.2.2.1a. The change in the mean dry weight of excised *Cuscuta campestris* shoot tips with time cultured in three different liquid nutrient media. (○) = water, (●) = Basal medium (■) = Basal medium plus GA_3 (⊥) = standard error bars



sharply between days 6 and 8 then rose again by day 14.

The mean dry weight of the shoot tips cultured in BMGA decreased between days 1 and 2 before an increase between days 2 and 3. This was followed by a small decrease between days 3 and 5 and then a large increase between days 5 and 6. There were further increases in the mean dry weight between days 6 and 7 and 7 and 14.

The mean dry weights of shoot tips cultured in BM or BMGA were not significantly different from each other after 14 days. The mean dry weight of shoot tips cultured in water was however significantly smaller than either of the mean dry weights of shoot tips cultured in BM or BMGA.

The change in fresh weight (Fig 2.2.1b)

The mean fresh weight of shoot tips cultured in water increased between days 0 and 1 and then decreased between days 1 and 2. This was followed by an increase between days 2 and 3 then another decrease between days 3 and 4. There was a large increase between days 4 and 5 followed by a decrease between days 5 and 6. After this there was another increase between days 6 and 7 before a decrease between days 7 and 14. The mean fresh weight of shoot tips cultured in BM increased on successive days from day 0 to 3. The mean fresh weight then decreased between days 3 and 5 and days 5 and 6, remaining the same between days 6 and 8 before increasing again between days 8 and 14. The mean fresh weight of shoot tips cultured on BM supplemented with GA_3 were larger than the mean values for feshly excised tips on all the days measurements were taken. The mean fresh weight increased between day 0 and 1, then decreased slightly between days 1 and 2 before a large increase between days 2 and 3. After this the mean fresh weight decreased between days 3 and 5 before rising again between days 5 and 6 and 6 and 7. The mean fresh weight decreased between days 7 and 14.

The change in length (Fig 2.2.1c)

The mean length of shoot tips increased when they were cultured in water. The mean length increased between days 0 and 1 with a small decrease between days 1 and 2 before increases on successive days between days 2 and 5. Then the mean length decreased between days 5 and 6 before a small rise between days 6 and 7. Finally the mean length decreased slightly between days 7 and 14.

The mean length of shoot tips cultured on BM increased on successive days between days 0 and 3 before a decrease between days 3 and 5. The mean length then increased between days 5 and 6 with a small decrease between days 6 and 8 before a large increase between days 8 and 14.

Fig. 2.2.Ib. The change in the mean fresh weight of excised *Cuscuta campestris* shoot tips with time cultured in three different liquid nutrient media. (○) = water, ( ) = Basal medium and (■) = Basal medium plus GA_3 (⊥) = standard error bars.

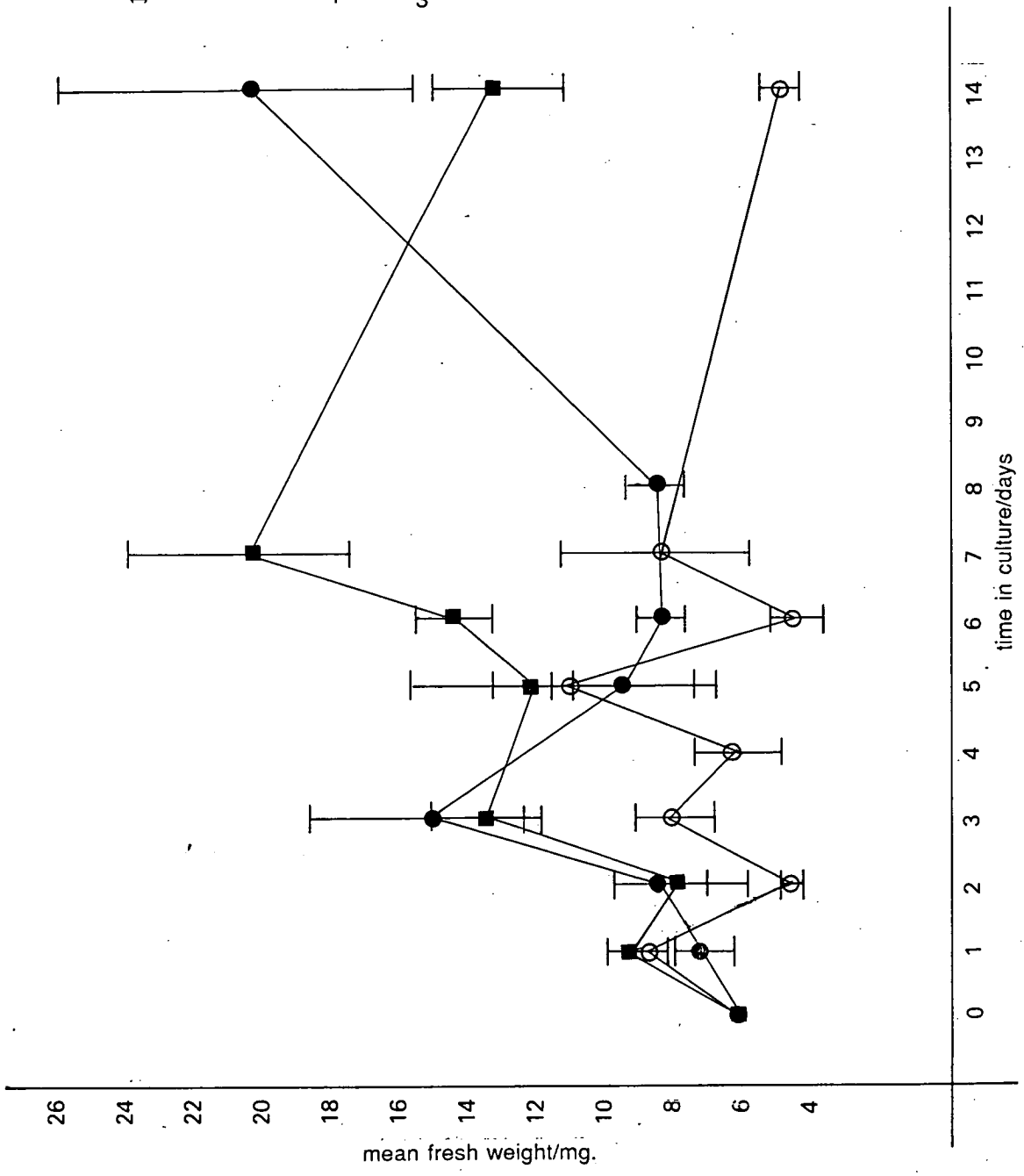
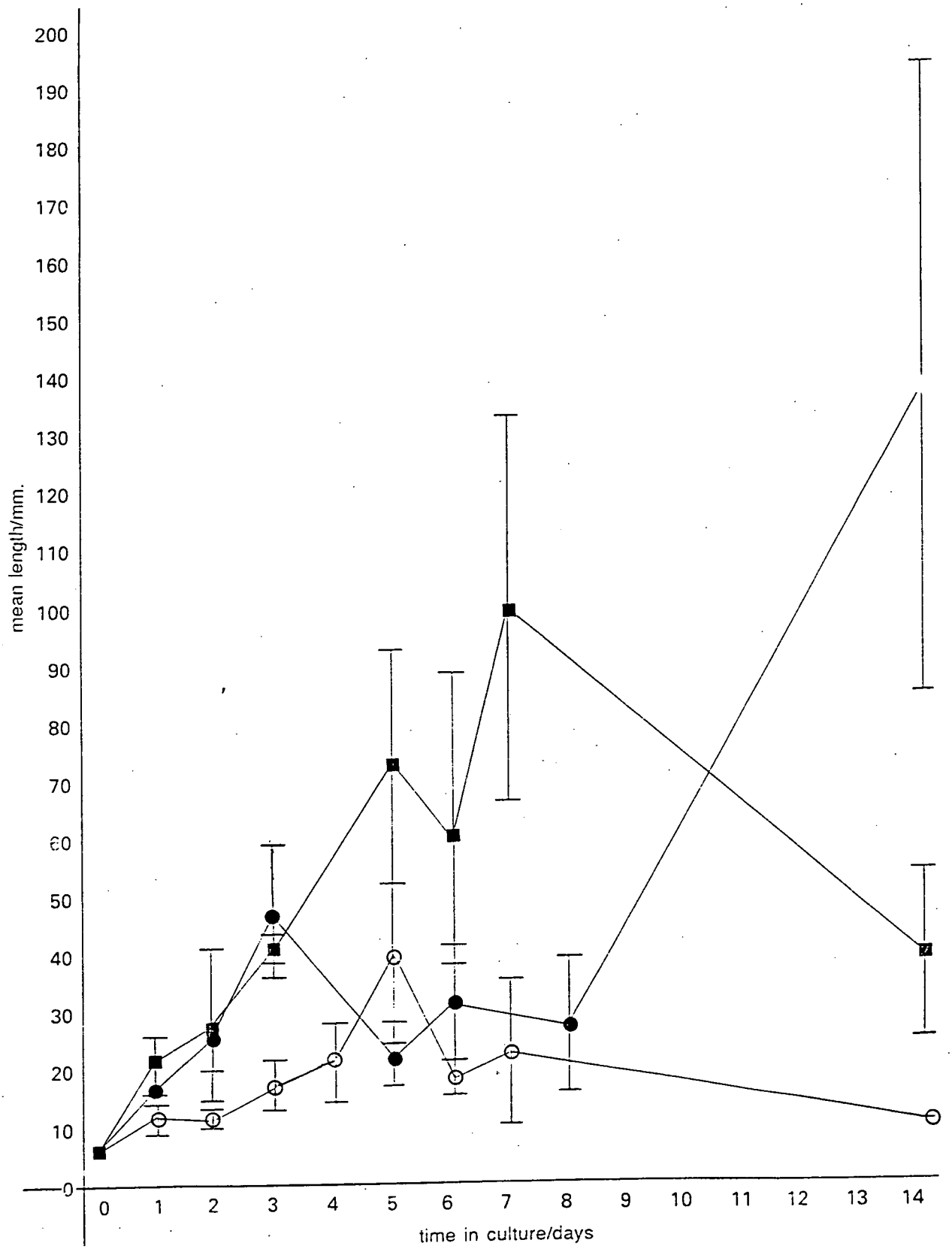


Fig. 2.2.Ic. The change in the mean length of excised *Cuscuta campestris* shoot tips with time cultured in three different liquid culture media. (○) = water, (●) = Basal medium and (■) = Basal medium plus GA_3 (I) = standard error bars.



The mean length of shoot tips cultured in BMGA increased on all the days on which measurements were taken from day 0 to 5.

The mean length then decreased between day 5 and 6 before a further increase between days 6 and 7. Finally the mean length decreased greatly between days 7 and 14.

The experiment showed the following

(1) Excised sterilised shoot tips cultured with nutrient medium BM or BMGA increase in dry weight over a 14 day period.

(2) Excised sterilised shoot tips cultured with nutrient medium increase in fresh weight more than shoot tips cultured in water over a 14 day period.

(3) Excised, sterilised shoot tips placed in water or liquid culture medium will increase in length. Over a 14 day period those cultured in nutrient medium elongate to a greater extent than those in water.

The increases in the dry weights of shoot tips in this experiment implies that they were able to assimilate components of the liquid culture medium. It is possible that the parasite could be labelled via the culture medium.

Sterilisation, Culture and Coiling

It was intended to inoculate cultured parasite onto cultured host *in vitro* . It was shown that the parasite could be cultured. However, it was not known if the culture procedure affected the ability of the shoot tips to coil and infect. The next step was to see if the parasite shoot tips which had undergone a sterilisation procedure and had then been placed in culture medium retained their ability to respond to a host plant by coiling and invading the host. Accordingly an experiment was performed to determine whether the culture procedure adopted changed the ability of shoot tips to coil around and infect the host.

2.2.2. The effect of the culture procedure on the ability of excised *Cuscuta* shoot tips to coil and infect

The aim of this experiment was to see how culturing shoot tips affected their ability to infect.

Experimental

Each day for 4 days ten 50mm *Cuscuta campestris* shoot tips were excised, sterilised and then trimmed to 40mm before being cultured as described in section 3b of Ch2. On the fifth day all the cultured shoot tips were harvested and inoculated along with 10 freshly excised 40mm shoot tips on to petioles of greenhouse-grown *Pelargonium* plants. Each tip was observed daily for evidence of coiling, prehaustoria, papillate appendages and tip advance. The proportion of replicates of each type of shoot tip which achieved particular

Table 2.2.2a The frequencies with which 40mm shoot tips cultured for 0 to 4 days achieved the various stages of infection after being inoculated onto *Pelargonium* petioles *in vivo*

Days in Culture	Replicates	% Coiling	% Forming prehaustoria	% Forming papillate appendages	% achieving tip advance
0	10	70	60	50	50
1	9	100	100	77.8	55.6
2	7	57	57	42	42
3	8	100	100	62.5	62.5
4	8	62.5	50	37.5	25

stages are shown in Table 2.2.2a.

The proportions of inocula reaching each stage of infection

All the shoot tips which were cultured increased in length. Shoot tips which were cultured for I and 3 days coiled around the host in all cases. Those not cultured and those cultured for 2 and 4 days coiled in 70, 57 and 62% of cases respectively. All the coiled regions of shoot tips which had been cultured for I and 3 days went on to form prehaustoria. Those not cultured at all went on to form them in only 60% of cases. Shoot tips cultured for 2 and 4 days formed prehaustoria in 57 and 50% of cases respectively. The number of replicates which reached the next stage decreased for all the treatments. The shoot tips cultured for I day developed papillate appendages in 77.8% of cases. Those cultured for 2, 3 and 4 days developed them in 42, 62.5 and 37.5% of cases respectively. Half of the uncultured petioles reached this stage. Finally the proportion of shoot tips reaching the tip advance stage for those cultured for I and 3 days is 55.6 and 62.5% respectively. Half the freshly excised shoot tips reached this stage and 44 and 25% of those cultured for 2 and 4 days respectively.

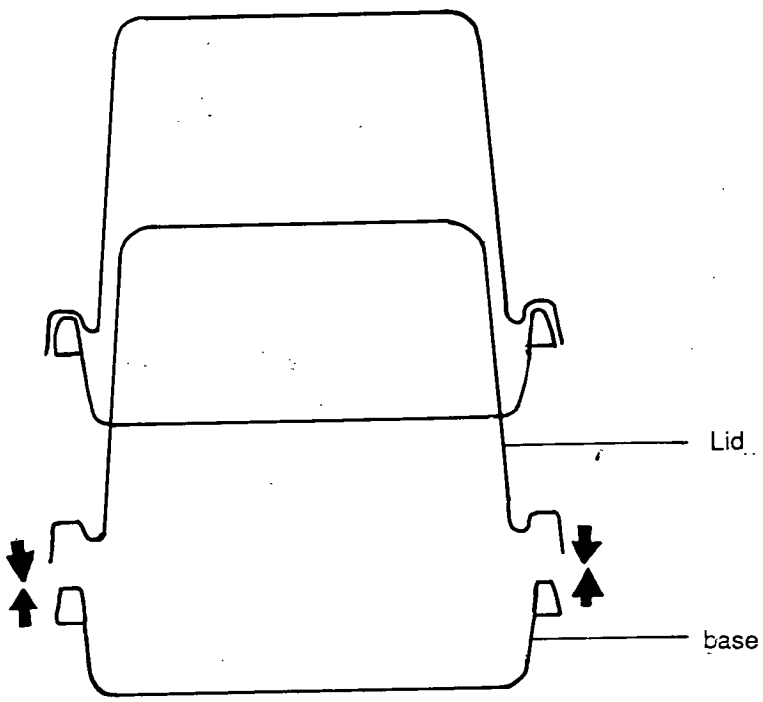
This experiment showed that the sterilisation procedure did not remove the ability of the shoot tips to coil and infect. One-day-old cultured shoot tips were more infective than freshly excised shoot tips. Shoot tips cultured for 3 days prior to inoculation performed best of all and those cultured for four days least well. From this experiment it may be concluded that shoot tips could be cultured for at least 3 days without reducing the ability to coil and produce a successful infection. Whether this is true for longer periods is not known, as this experiment was not extended or repeated. Attention was now turned to the culturing of the host plant.

2.3 The culture of the host

Parasite shoot tips had been successfully cultured and it was shown that in the short term, ability to coil was not removed by the procedure. These cultured shoot tips were to be inoculated on to cultured *Pelargonium* petioles in a sterile environment. This environment was provided by a 2-piece plant container (Flow laboratories , see Fig 2.3a).Assembled plant containers provide an airtight sterile culture chamber with an approximate internal volume of I300cm^3.

Cuscuta infection is initiated by the parasite coming into contact and coiling around a vertical component of the host (see section I). It was necessary for the host petioles to be cultured vertically and to have a free space around the complete circumference to allow the parasites to coil around them. This was the reason for using the plant containers. The culture procedure was as follows:

Fig. 2.3a. A diagram illustrating the two piece sterile plant containers used to house *Cuscuta/Pelargonium in vitro* interactions.



Preparing the plant containers

The first step was to prepare the plant containers. The presterilised tops and bottoms of the 2-piece containers were assembled inside a Laminar flow cabinet. The material from which the containers were made is an extremely poor electrical conductor. As a result their surfaces became electrostatically charged. Any *Cuscuta* shoot tips which were placed inside the containers were attracted to the charged surfaces and became stuck to the inner walls, preventing any interaction between the host the the parasite. To overcome this problem the outer surfaces were sprayed with a conductive liquid supplied by R.S. Components Ltd. (anti-static spray). When the surface was completely dry approximately 200ml of molten agar nutrient medium (M and S medium plus 30 $g.l^{-1}$ sucrose, 0.2 $mg.l^{-1}$ kinetin and l% Oxoid No. 3 agar) was poured into the base of each container and left to solidify.

Preparing petioles for culture

Sixty millimetre lengths of petioles from expanding *Pelargonium* leaves were excised, waxed and sterilised as described in section 3.3. of Ch2. Once sterilised the waxed ends were removed and the petioles were trimmed to 50mm with a scalpel. The cut stem ends of the petioles were placed into the solidified nutrient medium in the base of the plant container. In early experiments the cut laminal end was left uncovered. However, this led to drying out from the top and rapid senecence of the petiole (see Fig 2.3b). Addition of l% IAA in lanolin paste was found to delay the onset of senescence of the petiole. Fig 2.3c and d show that the addition of lanolin paste to the top of petioles prevented them drying out. However the petioles still went red, showing symptoms of senescence. When IAA was added to the paste the petioles remained green for prolonged periods. Subsequently it was decided to supply the cut laminal end with IAA dissolved in a unit amount of agar nutrient medium. A concentration of 2mg per litre was found to cause severe disorganisation of the whole petiole (callusing). The concentration was changed to 0.2mg per litre. The cut laminal ends were covered with Taab embedding capsules containing 0.8 ml of Agar nutrient medium as shown in Fig 2.3e. The capsule was supported in place by a glass rod. Other constituents of the medium were taken from Parkinson and Yeoman (1982) where internodes were cultured in Petri dishes.

The petiole nutrient medium

The content of the growth medium in base and capsules was the subject of several experiments which are described below.

A culture medium was required which could maintain excised petioles in the same physiological state as in the intact plant. It was important to establish that petioles did not begin to senesce when they were excised and cultured. Senescence in green tissues is

Fig. 2.3.b-d. The changes in excised *Pelargonium* petioles undergoing different treatments. (b) Excised petioles with their leaf ends pushed into growth medium while leaving their laminal ends exposed results in paling and shrivelling at the laminal ends. (c) Coating the laminal ends in lanolin paste prevents the shrivelling but does not prevent the petiole losing the green colour and turning red.
(d) Including l% 1AA in the lanolin paste prevents the loss of green colour and promotes some swelling of the laminal end.

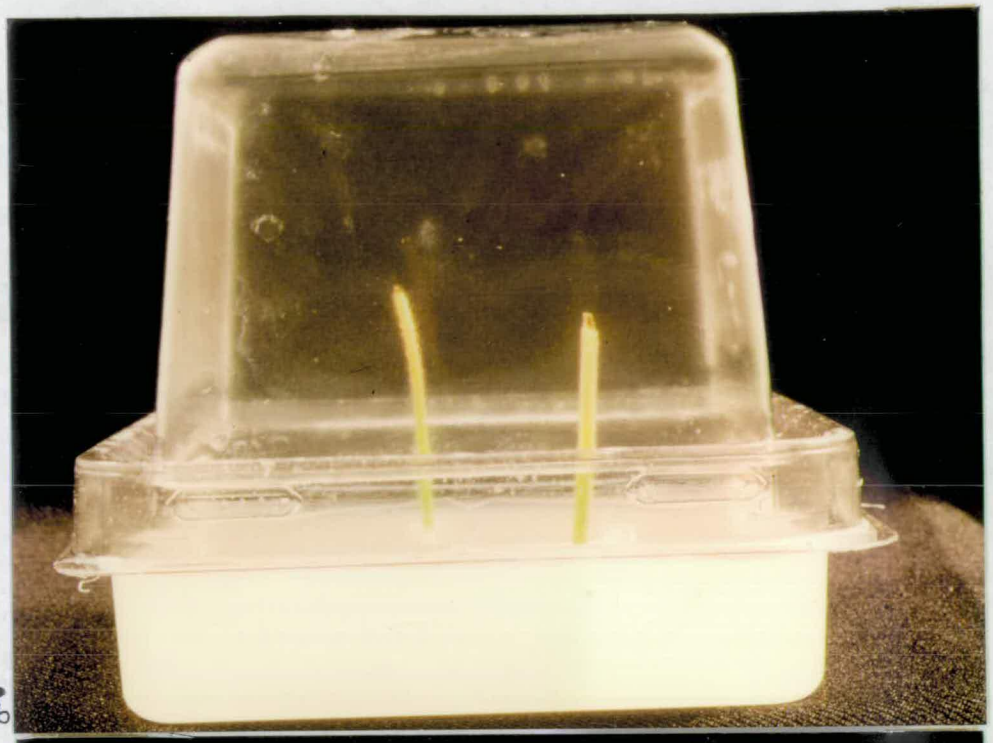


Fig. 2.3b

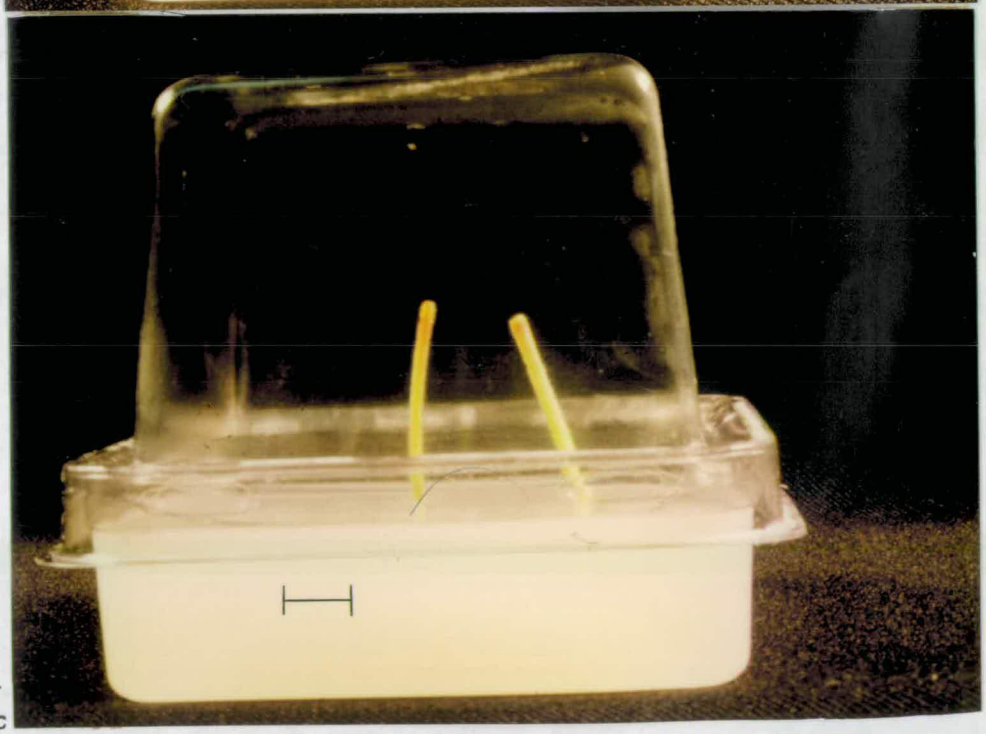


Fig. 2.3c

Fig. 2.3.d

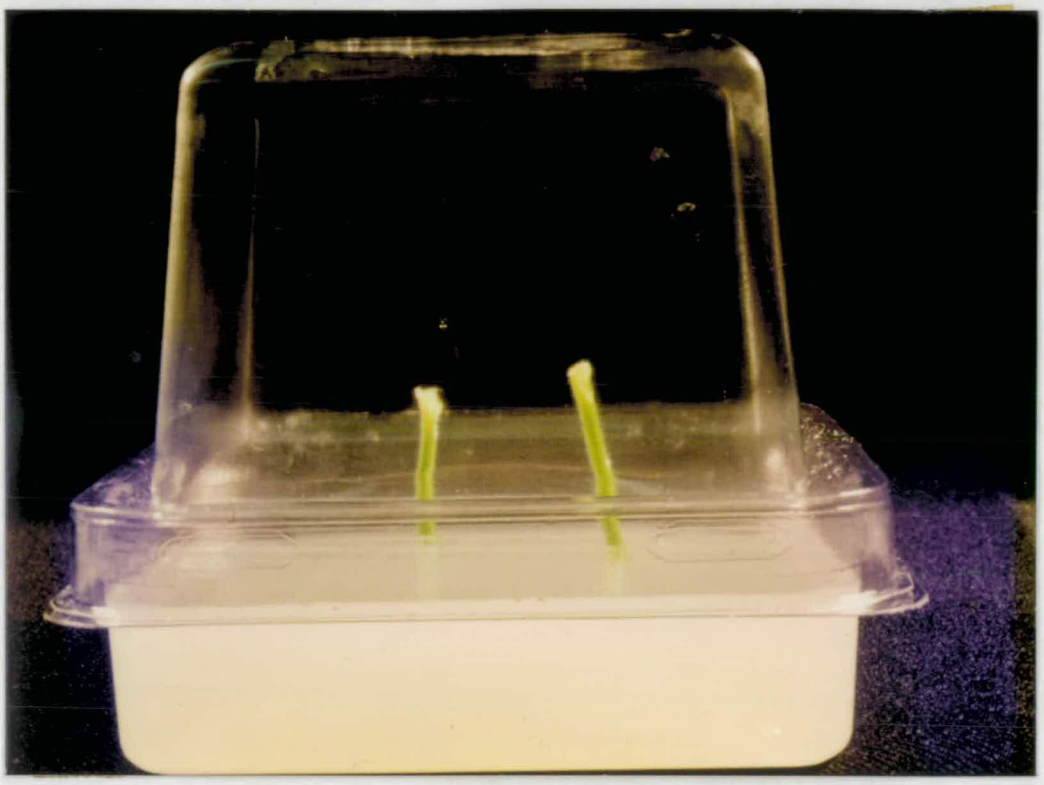
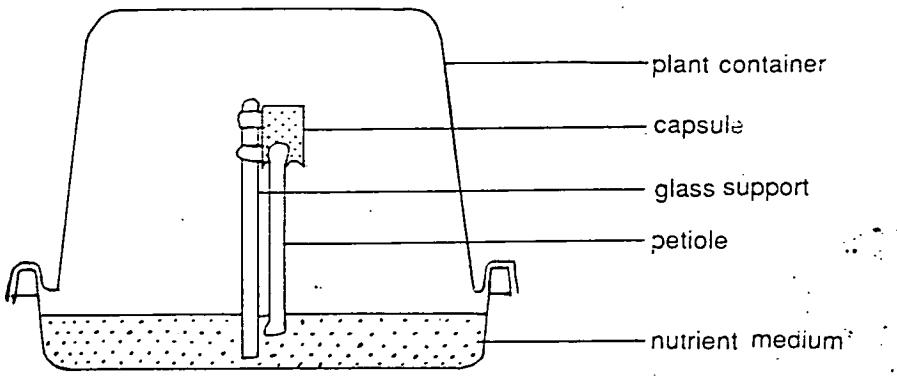


Fig. 2.3e. *Pelargonium* petioles are cultured with their cut stem ends immersed in the solid nutrient medium in the base of the plant containers. The laminal ends are covered with embedding capsules which also contain solid nutrient medium.



usually accompanied by a decrease in the chlorophyll (chl) concentration. In preliminary observations the presence of IAA appeared to delay the senecence of excised petioles. Attempts were made to quantify this effect. Petioles were cultured with or without a known concentration of growth substance in the capsule or base. Changes in chlorophyll content, fresh weight and dimension were monitored. The growth substance was supplied from the top only to set up a gradient within the petiole. This was thought in some way to mimick the situation in stems of whole plants.

2.3.1 The change in the fresh weight, and chlorophyll (chl) content of excised *Pelargonium* petioles when cultured with or without 0.2mg.l^{-1} IAA

The aim of the experiment was to determine how the chl content and the fresh weight of petioles changed with time in culture.

Experimental

This experiment was performed in August. Petioles were prepared for culture as described above. The IAA was filter-sterilised (see section 3.2 of Ch2) and added to the medium before it was syringed into the capsules. Half the plant containers contained petioles which had capsules with M and S plus 0.2mg.l^{-1} IAA, 0.2mg.l^{-1} kinetin and 2% sucrose. The other half had petioles with caps containing the same medium minus IAA. The medium in all the bases of the containers was the same as the capsule medium minus IAA. Four petioles were cultured in each plant container which were sealed with Parafilm before being transferred to a growth cabinet with constant fluorescent illumination of 10-15μ moles .$m^{-2.}$ $sec^{-1.}$ The temperature was maintained between 27 and 29.5^{o}C. In preliminary experiments the medium in some of the capsules dried out. To guard against this, capsules were replaced every 3 days.

On days 3, 6, 8 and 10 after the start of the experiment twelve petioles receiving each type of treatment were harvested. The fresh weights of each petiole were measured using an Oetling balance accurate to four decimal places. The 12 petioles from each treatment were grouped into 3 samples. The chl content of the samples was estimated by the method described in section 7.1 of Ch2. The concentrations of chla and b were calculated in mg per gram fresh weight and per petiole. The means and standard errors of chl contents and the fresh weights were calculated for each group of petioles. The chlb/chla ratio was also calculated. In senescing tissues the chla is known to degrade faster than chlb. A decrease in the value of the ratio implies that senescence is taking place. Analysis of variance was used to compare the weights and chl contents of petioles harvested at the same time but which had received different treatments.

Results - The change in the fresh weight of cultured petioles - (Fig 2.3.1a)

The mean fresh weight increased with time in culture whether IAA was supplied basipetally or not. The mean fresh weight of petioles cultured with IAA decreased slightly between days 0 and 3 before progressive increases between days 3 and 6, 6 and 8 and 8 and 10. The mean fresh weight of petioles cultured without IAA also decreased between days 0 and 3. After this it increased between days 3 and 6 before a slight fall between days 6 and 8. There was then a further increase between days 8 and 10. The mean fresh weights of petioles cultured with IAA were larger than those for petioles not receiving IAA on all the days samples were taken. However, none of these differences were significant at the 5% level.

The change in the mean chla and chlb concentration (mg per g fresh weight) (Fig 2.3.1b).

The mean chla concentration of petioles cultured with IAA decreased between days 0 and 3 before increasing again between days 3 and 6. After this the mean concentration decreased between days 6 and 8 but remained constant between days 8 and 10. The mean chla concentration in petioles cultured without IAA remained oonstant between days 0 and 3 and then progressively decreased between days 3 and 6, 6 and 8 and 8 and 10.

The mean chlb concentration of petioles cultured with IAA decreased between days 0 and 3 before an increase between days 3 and 6. Then the mean concentration decreased between days 6 and 8 and slightly decreased between days 8 and 10. The mean chlb concentration for petioles cultured without IAA increased between days 0 and 3 before increasing again between days 3 and 6. The mean concentration rose again between days 6 and 8 before a fall between days 8 and 10. After 3 days the petioles cultured with IAA had a lower mean chla concentration than those which had not been supplied with IAA. The opposite was true after 6, 8 and 10 days of culture. These differences were significant at the 5% level, only after 8 days of culture. Petioles cultured with IAA had larger mean chlb contents per gram fresh weight after 6 and 8 days in culture and smaller mean values after 3 and 10 days. None of these differences were significant at the 5% level.

The change in the chl content per petiole (Fig 2.3.1c)

The mean chla content of petioles cultured with IAA decreased between days 0 and 3 before a large increase between days 3 and 6. This was followed by a small decrease between days 6 and 8 before a further increase between days 8 and 10. The mean chla content per petiole for petioles cultured without IAA decreased between all the days on which measurements were taken.

The mean chlb content of petioles cultured with IAA decreased between days 0 and 3

Fig. 2.3.1a. The change in the mean fresh weight of *Pelargonium* petioles cultured with or without 1AA (●) = with 1AA, (○) = without 1AA, (⌶) = standard error bars.

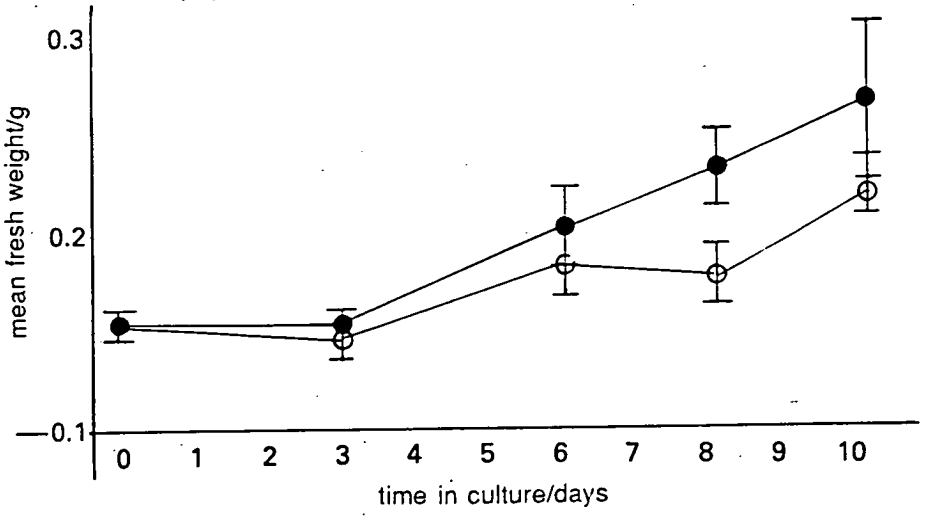


Fig. 2.3.1b. The change in the mean chlorophyll concentration with time for *Pelargonium* petioles cultured with or without 1AA. (●) = with 1AA (○) = without 1AA. (⌶) = standard error

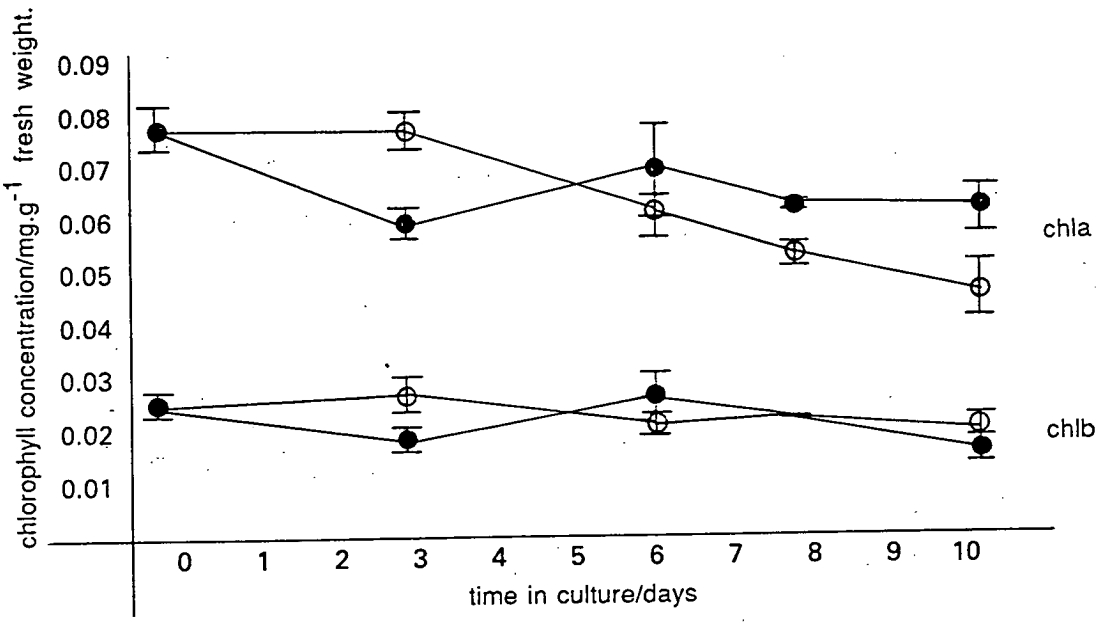


Fig. 2.3.Ic. The change in the mean chlorophyll content per petiole with time for *Pelargonium* petioles cultured with or without 1AA. (●) = with 1AA (O) = without 1AA. (I) = standard error bars.

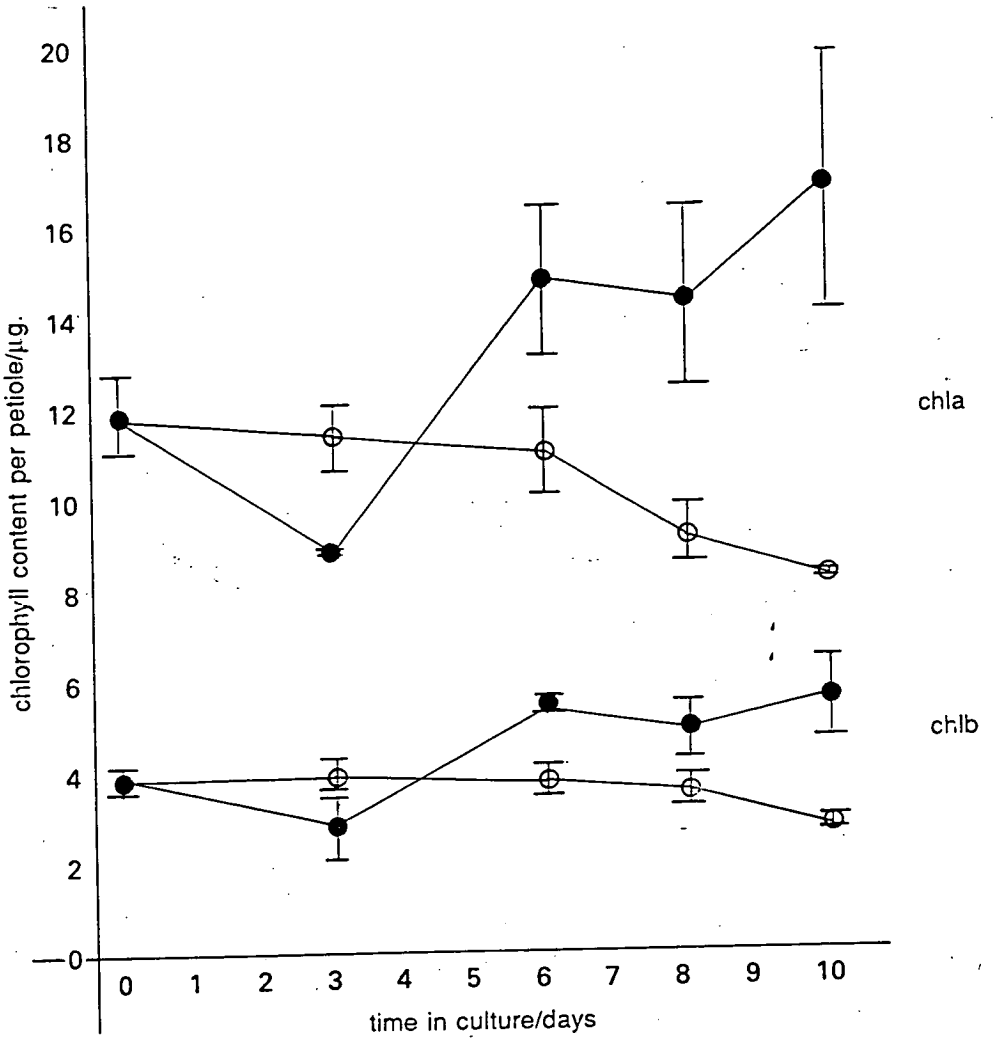
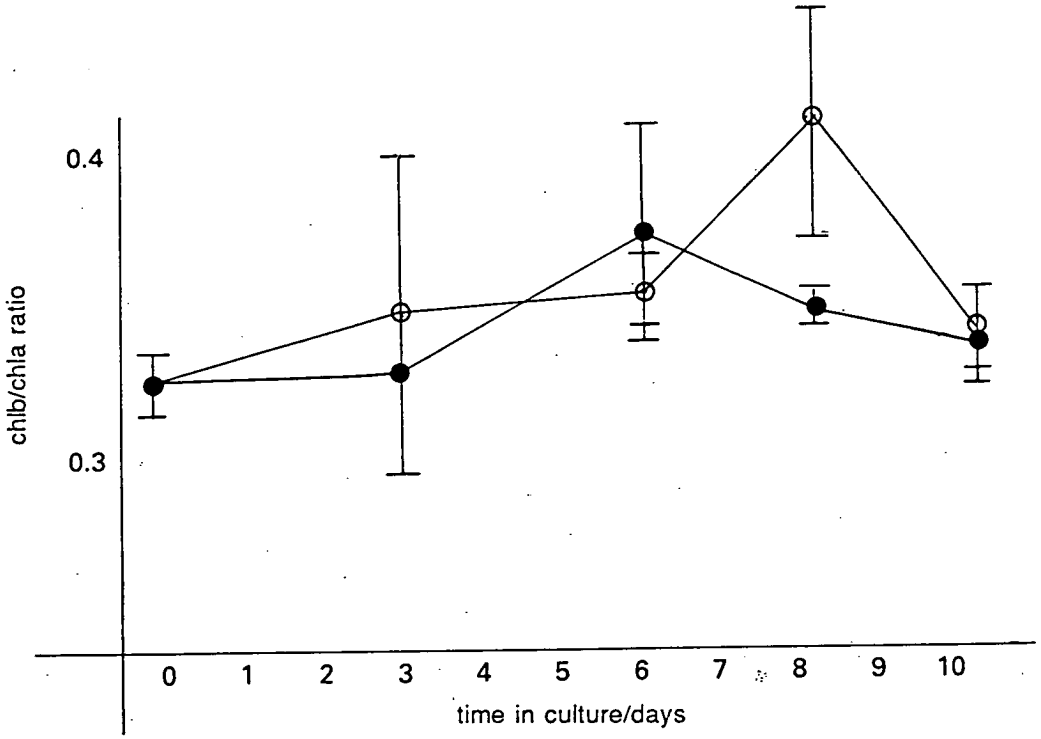


Fig. 2.3.Id. The change in the mean chlb/chla ratio with time for *Pelargonium* petioles cultured with or without IAA. (●) = with 1AA (○) = without 1AA. (I) = standard error



before increasing between days 3 and 6. Then there was a small decrease between days 6 and 8 followed by an increase between days 8 and 10. The mean chlb content of petioles increased slightly between days 0 and 3 before decreasing progressively between days 3 and 6, 6 and 8 and 8 and 10.

The mean values of chla per petiole for petioles cultured plus or minus IAA were not significantly different after 3 and 8 days in culture. They were significantly different at the 5% level after 10 days. The same was true for the mean values of chlb per petiole.

The change in the chlb/chla ratio (Fig 2.3.1d)

The mean chlb/chla ratio value for petioles cultured with IAA remained constant between days 0 and 3 before increasing between days 3 and 6. The mean ratio value then decreased again between days 6 and 8 and 8 and 10. The mean ratio value for petioles cultured without IAA increased between days 0 and 3, 3 and 6 and 6 and 8, then decreased sharply between days 8 and 10.

The ratios for petioles cultured with and without IAA were not significantly different from each other on any of the days measurements were taken.

This experiment has shown the following:-

(1) There were small increases in the mean fresh weights of petioles whether IAA was applied or not.

(2) The fresh weight of petioles cultured with or without IAA were not significantly different from each other on any of the days measurements were taken.

(3) The mean chla and chlb concentrations per unit fresh weight decrease with time under these culture conditions regardless of the presence of IAA.

(4) When IAA was supplied basipetally the mean chlorophyll content per petiole increased with time in culture and decreased for petioles without it. The mean chla and b contents per petiole were significantly different from each other after 10 days in culture.

(5) The mean ratios of chlb to chla for petioles cultured with or without IAA were not significantly different from each other on any days measurements were taken.

Having explored the effect on the chl content of supplying IAA basipetally, an experiment was performed to determine the effect of supplying IAA from both ends of the cultured petiole. For the sake of convenience the experiment was performed by culturing the petioles in Petri dishes.

2.3.2 The change in chlorophyll (chl) content and fresh weight of cultured petioles supplied with IAA from the top only and from both the top and bottom

The aim of the experiment was to determine whether cultured petioles would remain green

and healthy for longer if IAA was supplied from the top only or the top and bottom simultaneously. The experiment was performed with the Petri dish culture method described in section 3.8 of Ch.2.

Experimental

This experiment was performed in May. Approximately 144 petioles were excised, sterilised (see section 3.3 of Ch2) and then trimmed to 32mm before being inserted between the 2 layers of growth medium in the Petri dishes (see Fig 3.7ch.2 Each Petri dish contained three petioles. Half the petioles were cultured in Petri dishes which had the same medium in both halves (M and S plus 2% sucrose, 0.2mg.l^{-1} kinetin and (0.2mg.l^{-1} IAA). The other half of the petioles had this medium in only that part of the dish which would be at the top when the Petri dishes were mounted on the walls. The medium in the other half of these Petri dishes was the same except it contained no IAA.

Sixteen Petri dishes (8 of each treatment) were harvested 4, 8 and 11 days after the start of the experiment. Petioles from each treatment were individually weighed before being divided into three groups of four. The chlorophyll content was estimated for each group of four petioles as described in section 7.1 of Ch2. The chlorophyll concentration was calculated in mg per gram and the quantity per petiole in mg. The chl concentration (in mg per g) of freshly excised petioles was also calculated. Analysis of variance was used to compare the mean fresh weights, chl contents and chl concentration in petioles of the same age but receiving different treatments.

Results - The change in the mean fresh weight (Fig 2.3.2a)

The mean fresh weight of petioles cultured with IAA at the top only increased between days 0 and 4, 4 and 8 and 8 and 11. The mean fresh weight of petioles cultured with IAA at the top and bottom also increased between these days. By 4 days the mean fresh weights of petioles receiving either treatment were significantly larger (at the 1% level) than those at day 0.

After 4 and 8 days in culture the petioles which were supplied with IAA from both ends had larger mean fresh weights than those which received it only from the top. The opposite was true after 11 days. However none of these differences were significant at the 5% level.

The change in the mean chl concentration (Fig 2.3.2b)

The mean chla concentration of petioles supplied with IAA from both top and bottom decreased between days 0 and 4, 4 and 8 and 8 and 11. The same was true for petioles cultured with IAA only from the top. After 8 days both the mean chla and b concentrations in petioles supplied with IAA from the top and bottom were significatly different at the 5%

Fig. 2.3.2a. The change in the mean fresh weight of *Pelargonium* petioles with time cultured with 1AA supplied from the top and bottom or top only. ( ) = top and bottom (□) = top only, (I) = standard error bars.

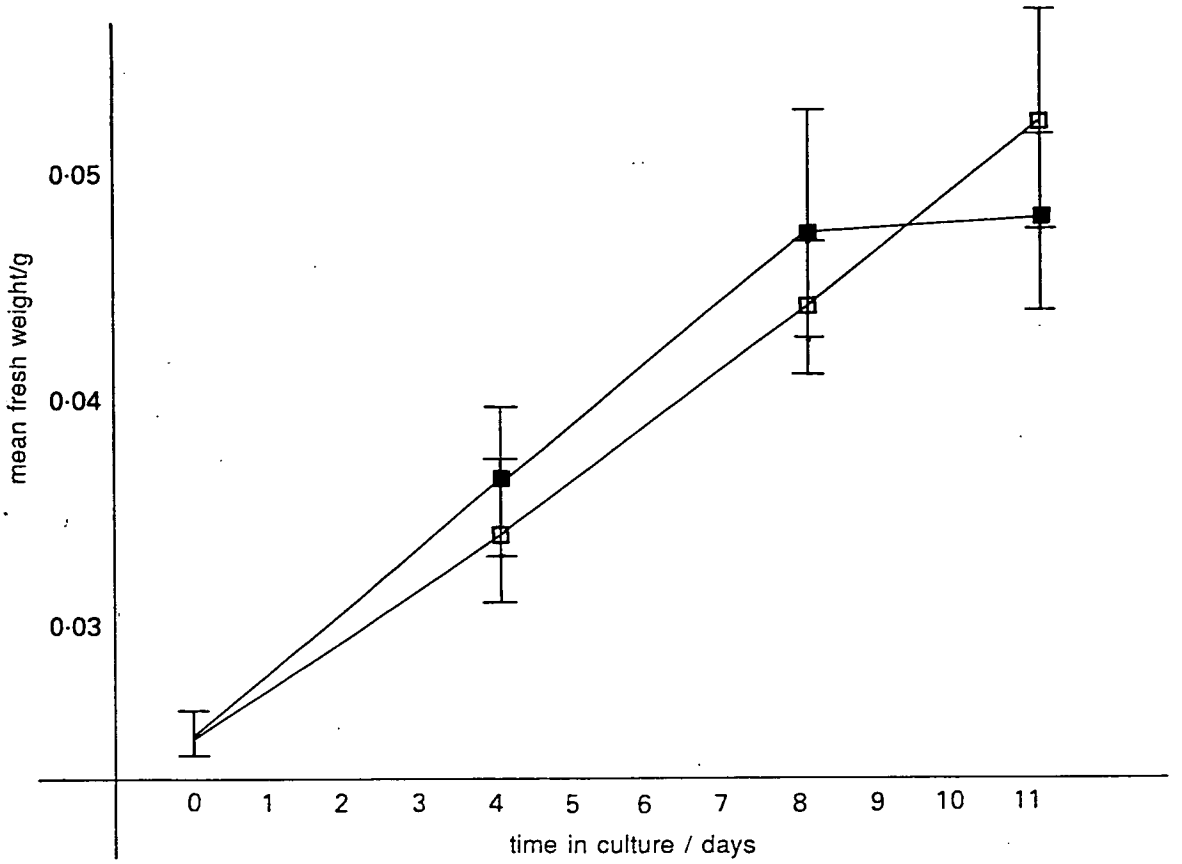
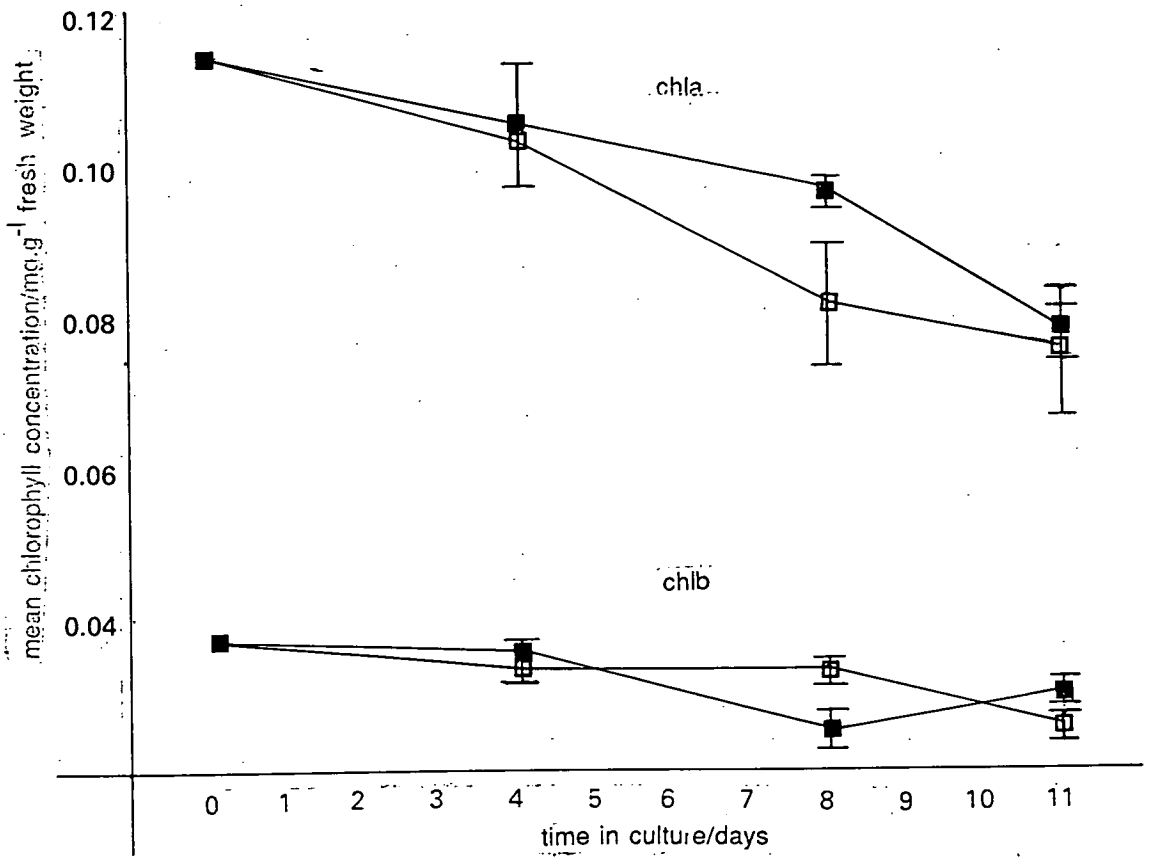


Fig. 2.3.2b. The change in the mean chlorophyll concentration of *Pelargonium* petioles with time when cultured with 1AA supplied from the top and bottom or top only. ( ) = top and bottom (□) = top only, (⌶) = standard error bars.



level from those for petioles at day 0. The mean concentrations for petioles supplied with IAA from the top only were not significantly different from those for petioles at day 0. The mean chla concentration after 11days for petioles supplied with IAA at the top and bottom was significantly different from that for petioles at day 0. The mean chlb concentration was not significantly different from that at day 0. After 11days the mean chla and b concentrations in petioles supplied with IAA from the top were significantly different (at the 5% level) from these for petioles at day 0. The values for petioles supplied with IAA from both ends were less than those for petioles supplied with IAA only from the top on all days on which measurments were made. However, none of the differences were significant at the 5% level. The mean chlb concentration (in mg per g) for petioles supplied with IAA from the bottom only decreased between days 0 and 4 and 4 and 8 before a small increase between days 8 and II. The mean chlb concentration for petioles cultured with IAA at both ends decreased between days 0 and 4 but remained constant between days 4 and 8 before falling again between days 8 and II. After 4 and II days the petioles which had been supplied with IAA from both ends had higher concentration than those in which it was supplied only from the top. The opposite was true after 8 days of culture. None of these differences were significant at the 5% level.

The change in the mean chl content (Fig 2.3.2c)

The mean chla content per petiole for petioles supplied with IAA from both ends increased between days 0 and 4 and 4 and 8, followed by a small decrease between days 8 and II. The mean chla content per petiole for petioles supplied with IAA from the top only increased sharply between days 0 and 4 and then more gently between days 4 and 8 and 8 and II. By 4 days the mean chla content for petioles cultured with IAA from the top only was significantly greater than that for petioles on day 0. On days 4 and 8 the mean chla contents of petioles supplied with IAA from both ends were greater than those where it was only supplied at the top. The mean contents were the same on day II. The differences found on days 4 and 8 were not significant at the 5% level.

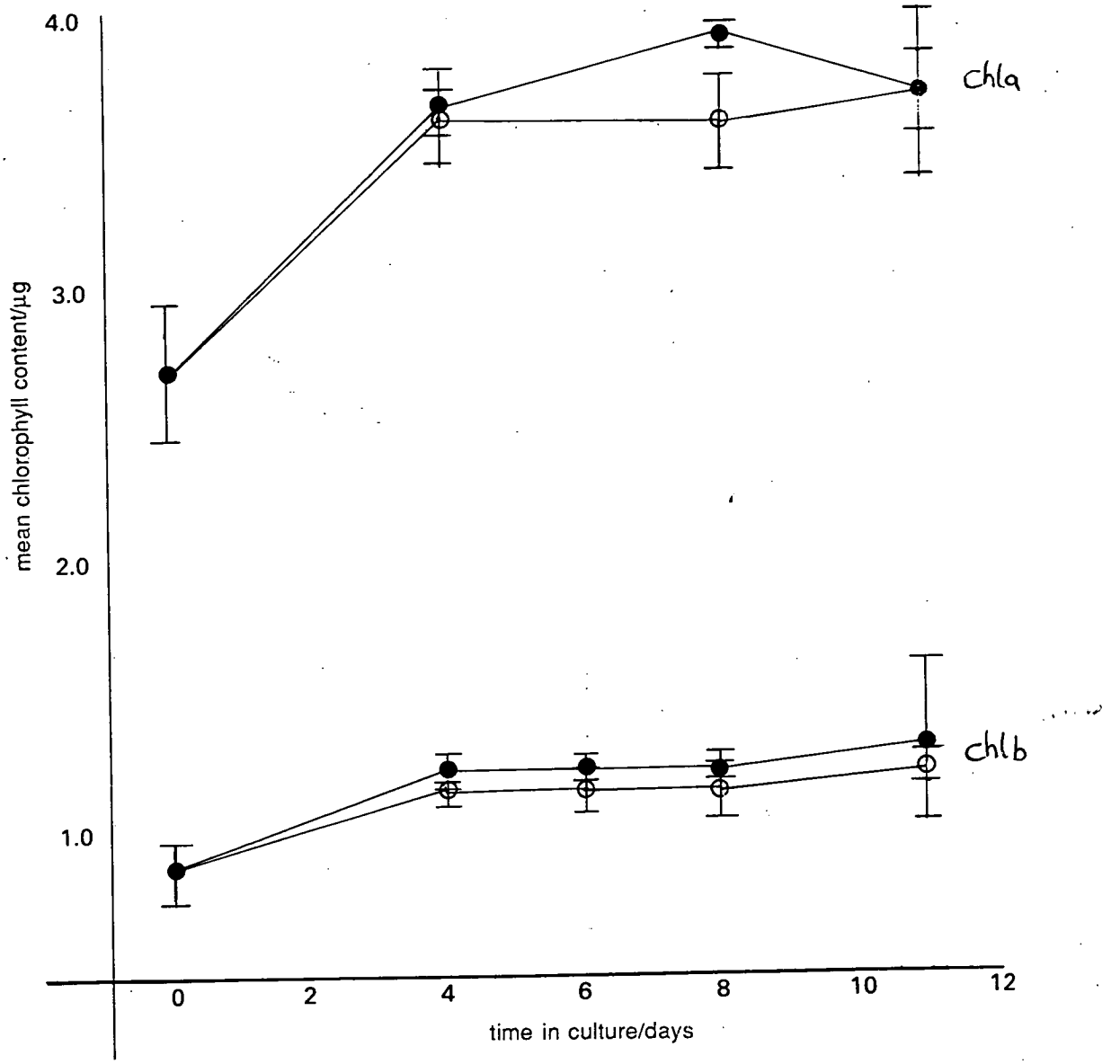
The mean chlb content for petioles cultured with IAA supplied at both ends increased between days 0 and 4, 4 and 8 and 8 and II. The same was true for petioles cultured with IAA only from the top. The mean content of chlb per petiole was greater for petioles supplied with IAA from both ends on all the days on which measurements were made. However none of these differences were significant at the 5% level.

This experiment showed:-

(I) There was no significant difference in the changes in fresh weight and chl concentration between petioles which were cultured with IAA supplied at the top only and both the top and bottom.

(2) There were significant increases in chla and chlb content between days 0 and 4 and

Fig. 2.3.2c The change in the mean chlorophyll content per petiole for *Pelargonium* petioles cultured with 1AA supplied from the top and bottom or top only (●) = top and bottom (O) top only,
(⊥) = standard error bars.



then no significant change between days 4 and II for petioles cultrured with IAA at the top only or top and bottom.

(3) Cultured petioles had significant increases in fresh weight between days 0 and 4.

(4) There was a significant overall fall in chla and chlb concentration between days 0 and 8 for petioles supplied with IAA top and bottom but not for petioles supplied with it only from the top.

It was decided to supply IAA only from the top in the culture system as there was no significant advantage in supplying it from the bottom as well.

Kinetin in the nutrient medium

Cytokinins are often used to delay the senescence of excised leaf tissues. Therefore kinetin was included in the culture medium supporting the *Pelargonium* petiole. Two experiments are described below which investigated the role of kinetin in delaying the senescence of cultured petioles. The concentration used was that used by Parkinson and Yeoman (1982) to support excised cultured internodes.

2.3.3 The change in the chlorophyll content, fresh weight and dimensions of *Pelargonium* petioles cultured with or without 0.2mg.l^{-1} kinetin

The aim of this experiment was to see if adding 0.2mg.l^{-1} kinetin to the nutrient medium delayed the decrease in chl concentration and promoted an increase in fresh weight for cultured petioles.

Experimental

This experiment was performed in February. Approximately seventy-two petioles were excised, sterilised and prepared for culture as described in section 2.3. Half the petioles were cultured with M and S plus 2% sucrose and 0.2mg.l^{-1} kinetin at their bases and the same medium plus IAA to a final concentration of 0.2mg.l^{-1} in the capsules. The other half were cultured with the same medium, except kinetin was omitted. After 3, 6 and I0 days four groups of three petioles receiving each treatment were harvested. For the petioles cultured for ten days without kinetin only 3 groups were taken (Fig 2.3.3a). Petioles were weighed individually and their lengths and diameters at the top, middle and bottom were measured to the nearest mm and 0.5mm respectively (see Fig 2.3.3b). The chl concentration was estimated as described in section 7.I of Ch2. The chla and chlb concentrations were calculated and expressed in mg per g fresh weight and in mg per petiole (see Fig 2.3.3.b). The fresh weights, dimensions and chl contents of freshly-excised petioles were also measured. Analysis of variance was used to compare the mean values of chl concentration, content, weight and dimension for petioles receiving different treatments.

Fig. 2.3.3a. The change in the mean fresh weight of *Pelargonium* petioles with time cultured with or without 0.2 mg l^{-1} kinetin. (●) = with kinetin (O) = without kinetin (I) = standard error bars.

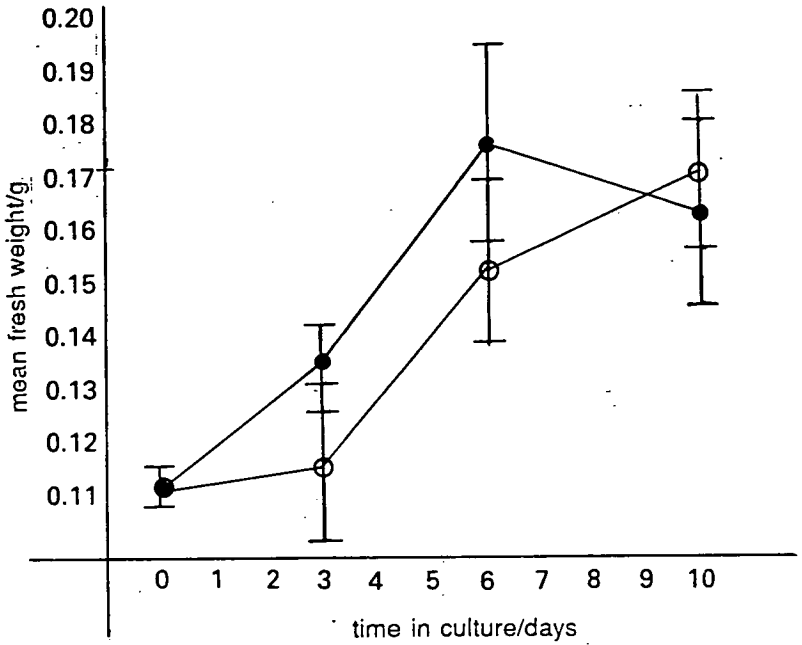
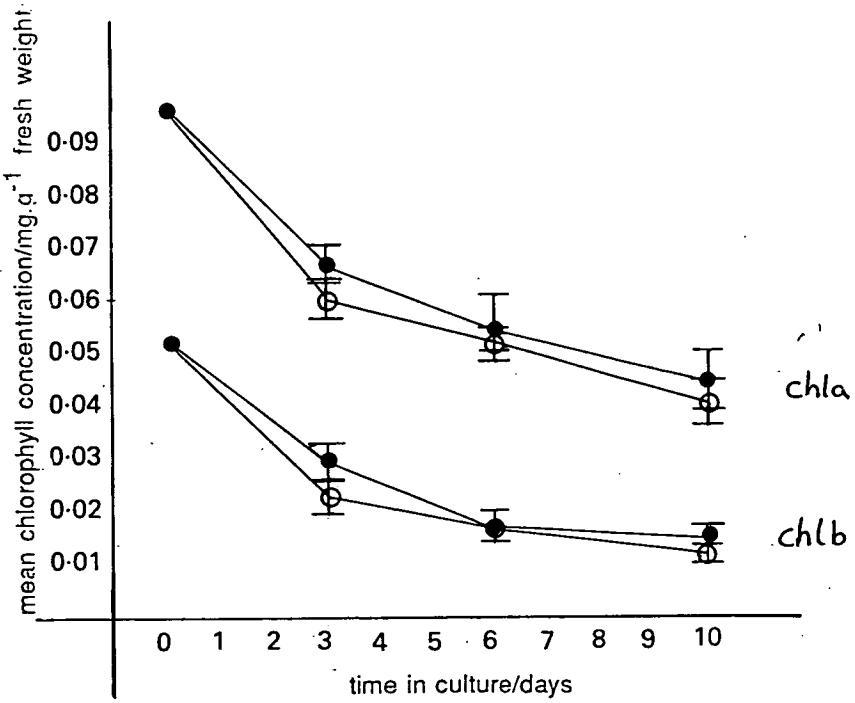


Fig. 2.3.3b. The change in the mean chlorophyll concentration of *Pelargonium* petioles with time when cultured with time cultured with or without 0.2 mg l^{-1} kinetin (●) = with kinetin (O) = without kinetin (I) = standard error bars.



The change in fresh weight (Fig 2.3.3a)

The mean fresh weight of petioles cultured with kinetin increased between days 0 and 3 and 3 and 6 before a decrease between days 6 and 10. The mean fresh weight of petioles cultured without kinetin also increased between days 0 and 3 and 3 and 6. By 6 days petioles cultured with or without kinetin had significantly larger mean fresh weights (at the 1 and 5% levels respectively) then freshly excised petioles. Then there was a further increase between days 6 and 10. The mean fresh weight of petioles cultured plus kinetin was significantly greater than at day 0 on days 6 and 10. The same was true for petioles cultured without kinetin. The mean fresh weight of petioles cultured for 6 and 10 days receiving different treatments were not significantly different from each other.

The change in the chl concentration (Fig 2.3.3b)

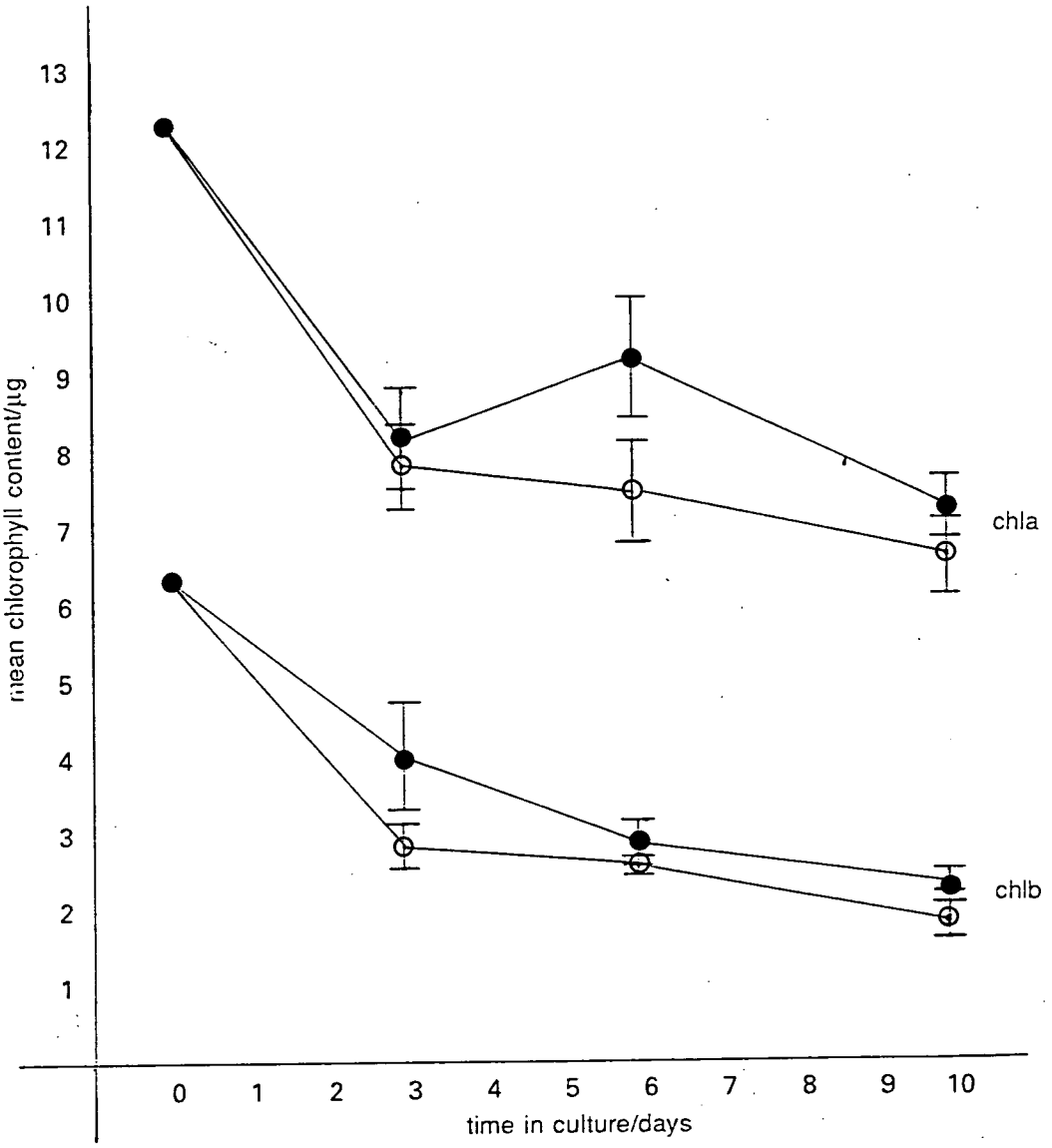
The mean chla concentration of petioles cultured with kinetin decreased between all the days measurements were taken, days 0 and 3, 3 and 6 and 6 and 10. The same was true for petioles which were not supplied with kinetin. The mean concentrations in petioles cultured with kinetin were larger than those cultured without it on all the days on which measurements were taken. However, none of these differences were significant at the 5% level. The mean chlb concentration of petioles cultured with kinetin decreased between days 0 and 3, 3 and 6 and also between days 6 and 10. The same was true for petioles cultured without kinetin. After 3 and 10 days the mean concentration in petioles cultured with kinetin was greater than that for petioles cultured without it. These differences were not significant at the 5% level. The mean chla concentrations in petioles cultured with or without kinetin for 3 days were significantly (1% level) smaller than those for freshly excised petioles. The same was true for the mean chlb concentration for petioles cultured without kinetin.

The chlb concentration in petioles cultured with kinetin for 3 days was not significantly different from that for freshly excised petioles. There was no significant difference in the chla and chlb concentration in petioles cultured for 3 and 10 days with kinetin. However the mean concentrations of both types of chl in petioles cultured without kinetin for 3 and 10 days were significantly different at the 1% level.

The change in the chl content per petiole (Fig 2.3.3c)

The mean chla content of petioles cultured with kinetin decreased between days 0 and 3, then increased between days 3 and 6 before another decrease between days 6 and 10. The mean chla content of petioles cultured without kinetin decreased between all the days on which measurements were taken, particularly between days 0 and 3. Petioles cultured with kinetin had higher mean chla contents on days 3, 6 and 10. These differences were not significant at the 5% level. The differences in the mean chla contents on days 3, 6 and

Fig. 2.3.3c. The change in the mean chlorophyll content per petiole with time for *Pelargonium* petioles cultured with or without 0.2 mg l^{-1} kinetin. (●) = with kinetin (○) = without kinetin. (⌶) = standard error bars.



10 between petioles receiving different treatments were not significant. The mean chla content of the freshly excised petioles was significantly larger (1% level) than those for petioles cultured for 3 days with or without kinetin.

The mean chlb content of petioles cultured with kinetin decreased between all the days measurements were taken. That for petioles cultured without kinetin decreased between days 0 and 3 with a further very small decrease between days 3 and 6 followed by another small increase between days 6 and 10. The mean chlb contents in petioles cultured with kinetin were greater than those without kinetin on days 3, 6 and 10. These differences were not significant at the 5% level. The overlapping of error bars shows there was no statistically significant fall in the mean chlb content between 3 and 10 days. The fall in the mean chlb content between days 0 and 3 for petioles cultured without kinetin was significant at the 1% level. The corresponding fall for petioles cultured with kinetin was not significant.

The change in the dimensions of petioles (Fig. 2.3.3d)

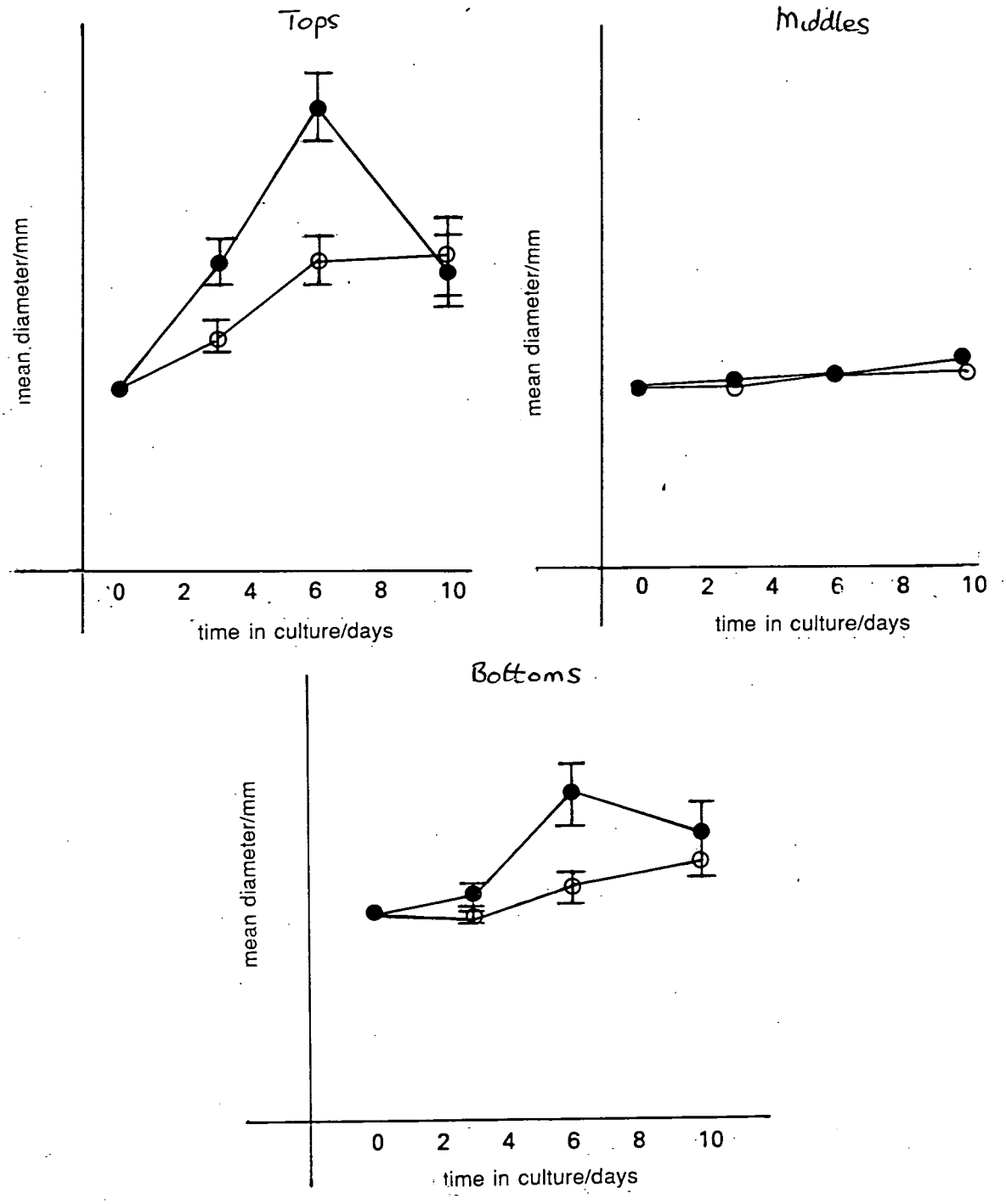
The change in the dimensions of the petioles cultured with or without kinetin did not change uniformly. The diameter of the tops and bottoms of the petioles changed more than the middle portions. The new tissues laid down during the expansion were white and not green like the original petioles had been.

Tops The mean diameter of the tops of petioles cultured with kinetin increased between days 0 and 3 and 3 and 6 then decreased between days 6 and 10. The mean diameter of this region in petioles cultured without kinetin increased between each day on which measurements were made. The increases in the diameter between 0 and 3 days for petioles cultured with and without kinetin are significant at the 1% level. The diameter of the tops of petioles cultured with kinetin for 3 and 6 days were significantly wider at the 1% level than those for petioles not cultured with kinetin.

Middles The mean diameter of the middle parts of the petioles cultured with kinetin increased only slightly between days 0 and 3, 3 and 6 and 6 and 10. The mean diameter of petioles cultured without kinetin increased slightly between days 0 and 3 and 3 and 6, then decreased slightly between days 6 and 10. There were no significant changes or differences in diameters between treatments.

Bottoms The mean diameter of bottom regions of the petioles cultured with kinetin increased between days 0 and 3 and 3 and 6 then decreased between days 6 and 10. The difference between days 0 and 3 were significant at the 1% level. The mean diameter of the bottom regions of petioles cultured without kinetin decreased between days 0 and 3 then increased between days 3 and 6 and 6 and 10. These petioles cultured without kinetin did not have a significantly larger diameter than freshly excised petioles until day 10. The

Fig. 2.3.3d. The change in the mean diameter of the top, middle and bottom portions of *Pelargonium* petioles with time when cultured with or without 0.2mg l^{-1} kinetin. ( ) = with kinetin (O) = without kinetin (I) = standard error bars.



mean diameters of the bottom regions of petioles cultured with kinetin were larger than those not cultured with kinetin on days 3, 6 and 10. These differences were only statistically significant (5%) on day 6.

This experiment showed the following:-

(1) *Pelargonium* petioles cultured in this way, with or without kinetin show significant increases in fresh weight.

(2) The chl concentration decreased significantly in the first 3 days of culture whether kinetin was added or not. Where kinetin was added there were no further significant decreases. Where it was not added significant decreases continued to occur.

(3) The chl content per petiole decreased significantly between days 0 and 3 and also between days 3 and 10 whether kinetin was added or not. When kinetin was present there was no significant fall in chlb content between days 3 and 10.

(4) The top region of the cultured petioles expanded during culture whether kinetin was present or not. Petioles cultured up to six days with kinetin in their culture medium had significantly larger diameters than petioles without it.

(5) The diameters of the middle regions of the petioles did not change significantly with or without kinetin in the culture medium.

(6) The diameters of the bottom regions of cultured petioles increased with time whether kinetin was present or not. The diameters of the petioles cultured with kinetin were significantly greater than those on day 0 by day 6. Those cultured without kinetin had a significantlty larger diameter by day 10.

The presence of kinetin seemed to prevent any significant change in chl concentration beetween days 3 and 10. However it did not prevent the significant fall of chla content between this period. There was some retention of chlb. It was decided to raise the concenteration of kinetin to see if this improved the situation.

2.3.4 The change in the fresh weight, dimensions and the chl content with time of excised petioles cultured with or without 0.4mg.l^{-1} kinetin

The aim of this experiment was to see if 0.4mg l^{-1} kinetin would delay changes in the chl content of cultured petioles.

Experimental

This experiment was performed in May. Approximately 120 petioles were prepared for culture as in the previous experiment except that a larger concentration of kinetin was used (0.4mg.l^{-1}). Half the petioles were cultured with medium containing kinetin in the base and in the capsules. After 3, 6 and 10 days forty petioles were harvested, twenty of which had been cultured with kinetin and twenty which had not. Their fresh weights

lengths and diameters at the top, middle and bottom were measured. Each sample of twenty petioles was then divided into four groups of 5, this gave 4 samples for each treatment on each day measurements were made. Chlorophyll assays were performed on each sample as described in section 7.I of Ch2. The chl concentration was determined in mg per g fresh weight and in mg per petiole.

Analysis of variance was used to compare the mean values of weight, dimension, chlorophyll content and concentration for petioles recieving different treatments. The chl concentrations and contents, weights and dimensions of freshly excised petioles were taken from the experiment in section 2.3.3.

The change in length (Fig 2.3.4a)

The mean length of petioles cultured with or without kinetin increased slightly between days 0 and 3, and 3 and 6 then decreased again between days 6 and l0. The lengths of petioles cultured with kinetin were slightly longer than those which did not have kinetin but these differences were not statistically significant.

The change in fresh weight (Fig 2.3.4b)

The mean fresh weights of petioles cultured with or without kinetin increased between days 0 and 3, and 3 and 6 before decreasing between days 6 and l0. The mean fresh weight of petioles cultured with kinetin was greater than that of petioles cultured without it on days 6 and l0. The opposite was true on day 3. However, none of the differences were significant at the 5% level. The increase in mean fresh weights of petioles cultured with and without kinetin between days 0 and 3 were significant at the 1% level. The overlap of the error bars for petioles cultured for 3 and 6 days shows that they are not significantly different from each other.

The change in diameter of the petioles (Fig 2.3.4c)

Tops The mean diameter of the top region of petioles cultured with kinetin increased between days 0 and 3 and 3 and 6 before decreasing again between days 6 and l0. The same was true for petioles cultured without kinetin. After 3 days petioles cultured with kinetin had a significantly larger (1% level) mean diameter than freshly excised petioles. Those cultured without it did not. By 6 days petioles cultured without kinetin did have a significantly larger diameter (1%). On days 3 and 6 the mean diameters of the tops of petioles cultured with kinetin were significantly larger than those which were cultured without it. The mean diameter of the top region for petioles cultured with kinetin were significantly wider than at day 0 after 3 days. The same was true for petioles cultured without it after 6 days.

Middles The mean diameter of the middle region of petioles cultured with kinetin increased slightly between days 0 and 3, then stayed the same between days 3 and 6 and 6

Fig. 2.3.4a. The change in the mean length of *Pelargonium* petioles with time when cultured with or without 0.4mgl^{-1} kinetin. (I) = standard error bars. ( ) = with kinetin (O) = without kinetin

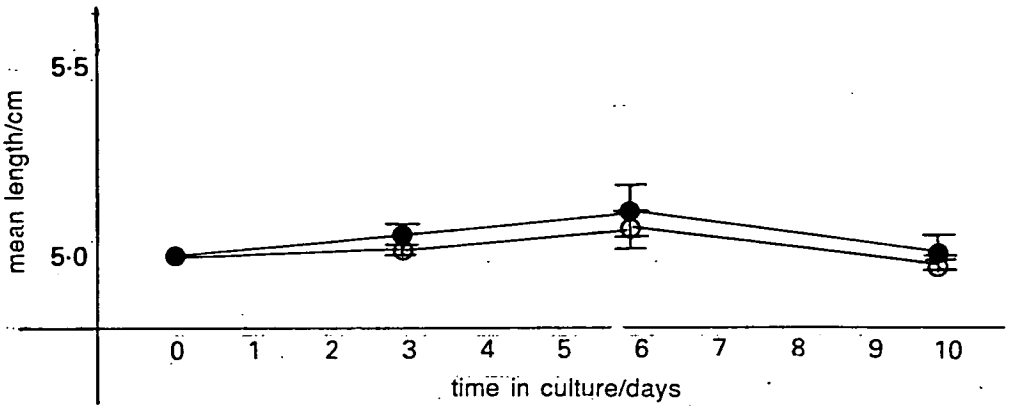


Fig. 2.3.4b. The change in the mean fresh weight of *Pelargonium* petioles with time when cultured with or without $0.4mgl^{-1}$ kinetin. (●) = with kinetin (○) = without kinetin (⊥) = standard error bars

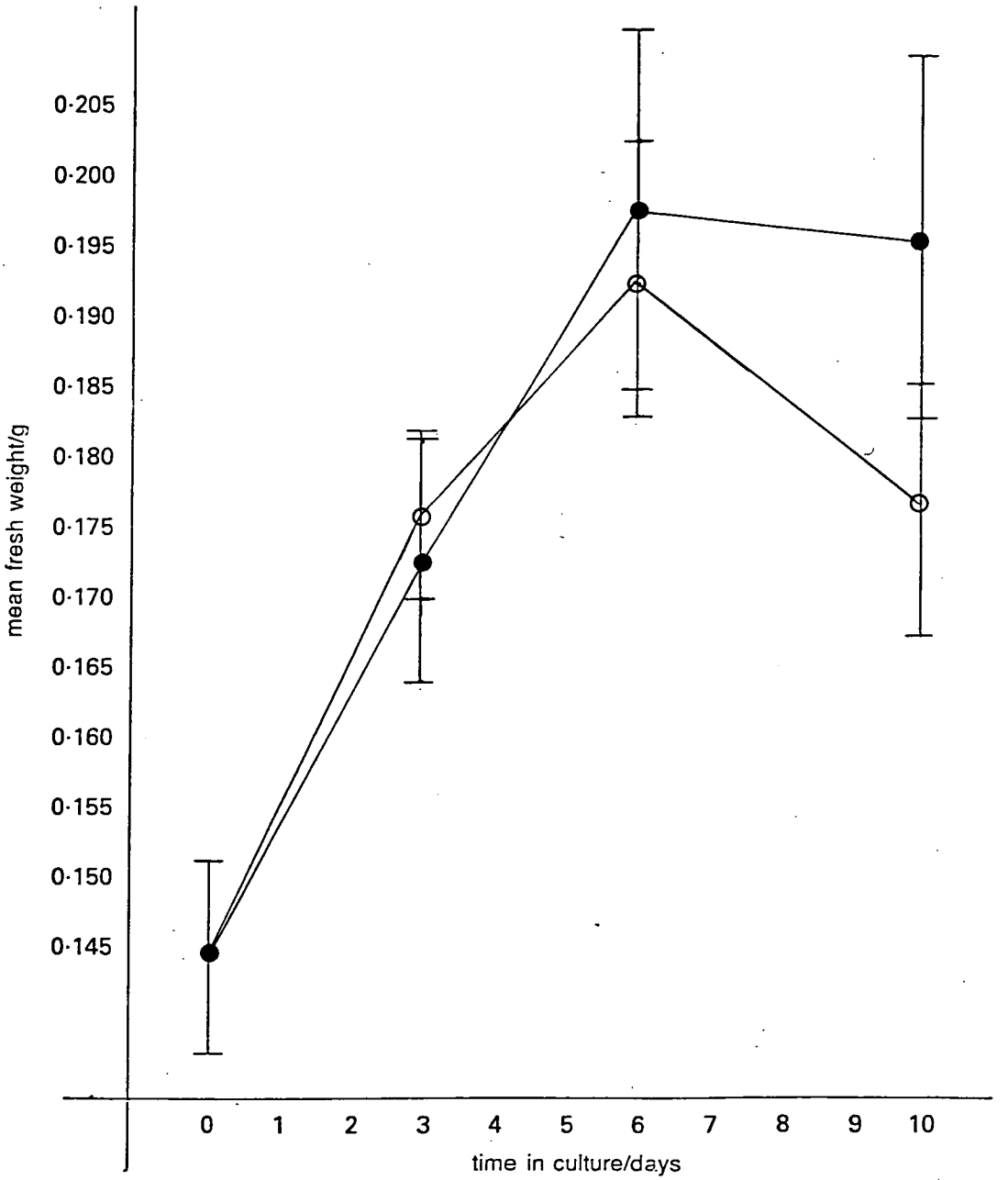
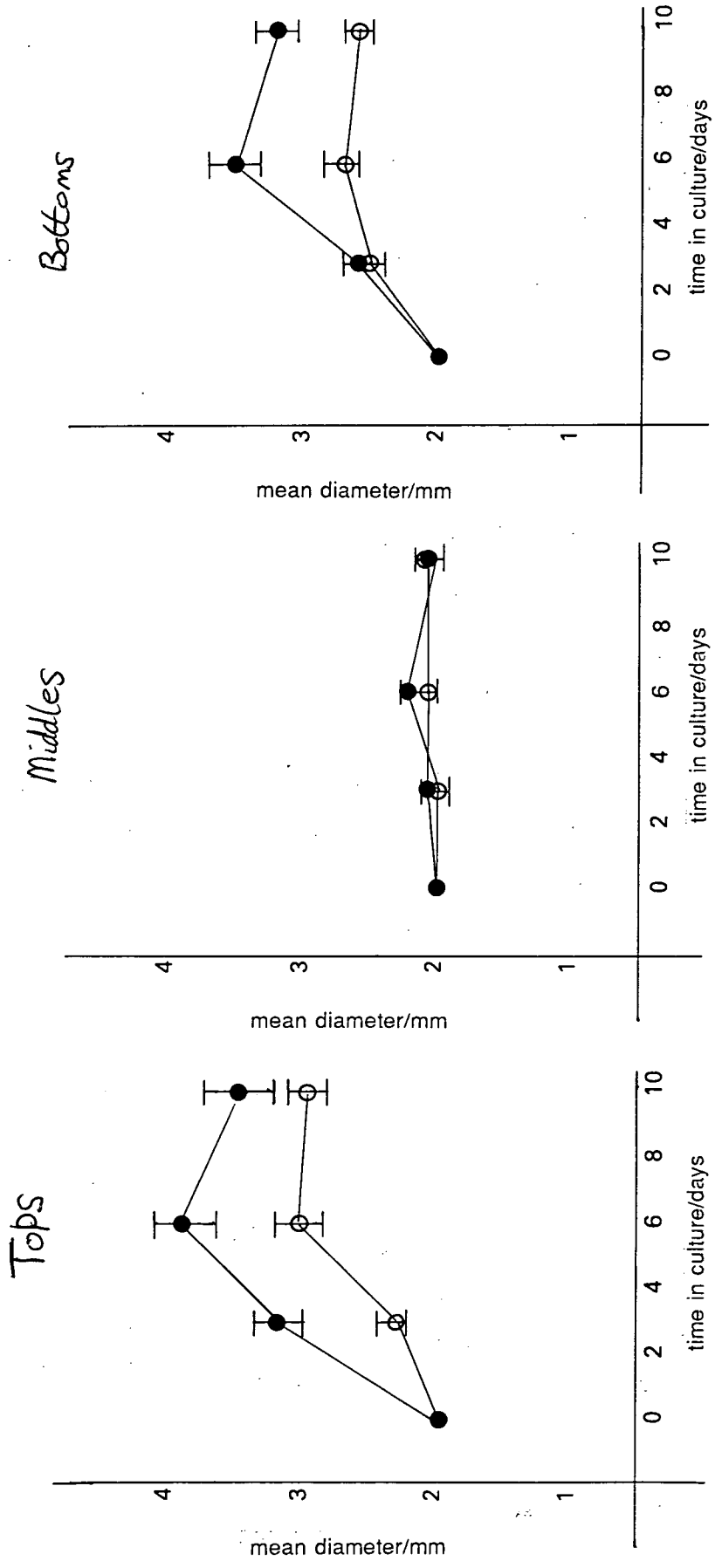


Fig. 2.3.4c. The change in the mean diameters of the top, middle and bottom portions of *Pelargonium* petioles with time when cultured with or without 0.4 mgl^{-1} kinetin ( ) = with kinetin (O) = without kinetin (⊥) = standard error bars.



and 10. That for petioles cultured without kinetin stayed the same between days 0 and 3, then increased between days 3 and 6 before decreasing again between days 6 and 10. The mean diameters of petioles cultured with or without kinetin were not significantly different from each other or from the diameter at day 0 on any day measurements were taken.

Bottoms The mean diameter of the bottom region of petioles cultured with kinetin increased between days 0 and 3, and 3 and 6 before decreasing between days 6 and 10. The same was true for petioles cultured without kinetin. After 3 days the mean diameters of the bottom regions of petioles cultured with or without kinetin was significantly larger (5% and 1% respectively) than that for freshly excised petioles. The mean diameter of petioles cultured with kinetin was significantly larger than that for petioles cultured without it on days 6 and 10 but not on day 3. By day 3 cultured petioles had significantly wider diameters than those at day 0.

The change in chl concentration (Fig 2.3.4d)

The mean chla concentration of petioles cultured with kinetin decreased between all the days measurements were taken. The same was true for petioles cultured without kinetin. The mean chla and chlb concentrations of petioles cultured for 3 days with or without kinetin were significantly smaller (1% level) than those for freshly excised petioles. The error bars for the mean chla concentrations in petioles cultured for 3 and 6 days with kinetin overlapped. The same was true for petioles cultured without kinetin. The error bars for the mean chlb concentrations in petioles cultured for 3, 6 and 10 days with kinetin overlapped. The same was true for petioles cultured without kinetin. Therefore there was no significant change between days 3 and 10. Petioles cultured with kinetin had larger mean chl concentrations than petioles cultured without it on days 3 and 6 but not on day 10. These differences were significant at the 5% level on day 3 only. The mean chla concentrations for cultured petioles were significantly smaller than that on day 0 after 3, 6 and 10 days.

The mean chlb concentration of petioles cultured with kinetin decreased between all the days measurements were taken. The same was true for petioles cultured without kinetin. The mean concentration of chlb in petioles cultured with kinetin was greater than that for those cultured without it on days 3 and 6. These differences were not significant.

The change in chl content per petiole (Fig 2.3.4e)

The mean chla content per petiole for petioles cultured with kinetin decreased between all the days measurements were made. The same was true for petioles cultured without kinetin. The mean chla contents of petioles cultured with kinetin were larger than those for petioles cultured without kinetin on days 3 and 6. These differences were significant

Fig. 2.3.4d. The change in the mean chlorophyll concentration of *Pelargonium* petioles with time cultured with or without 0.4 mgl^{-1} kinetin (●) = with kinetin (○) = without kinetin (⊥) = standard error bars.

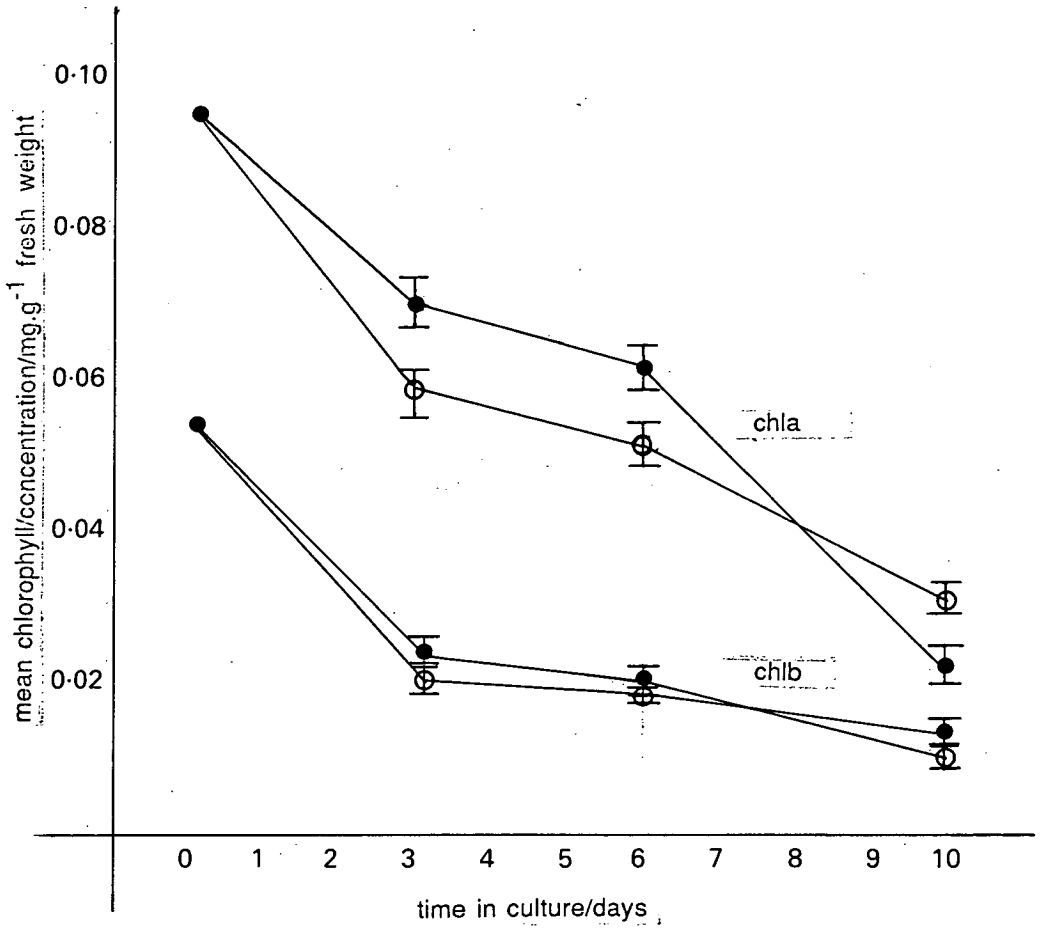
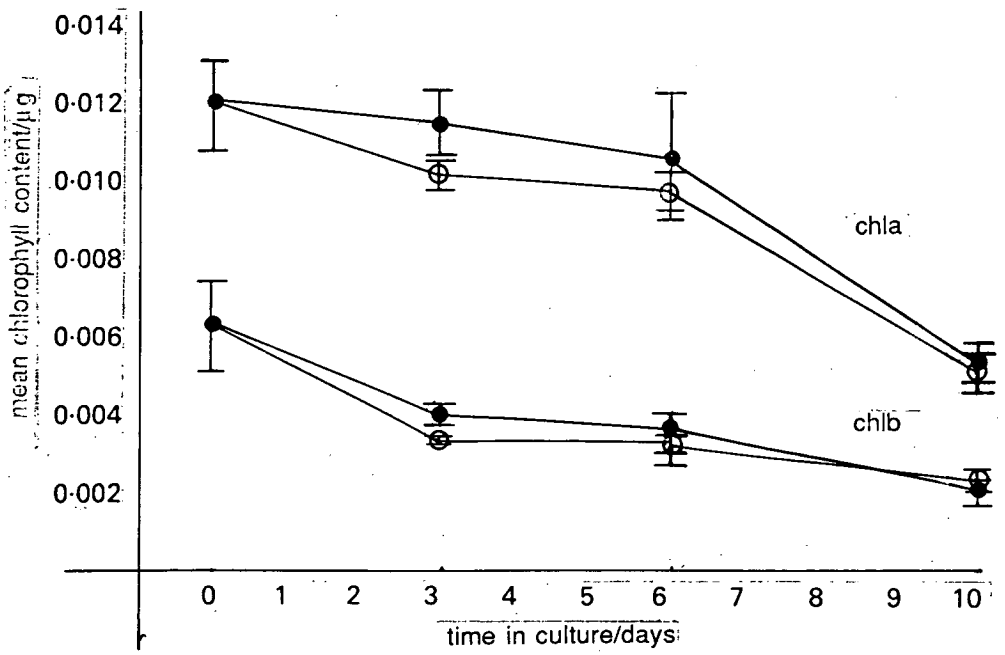


Fig. 2.3.4e. The change in the mean chlorophyll content per petiole for *Pelargonium* petioles cultured with or without 0.4 mgl^{-1} kinetin (●) = with kinetin (○) = without kinetin (⊥) = standard error bars.



only after 3 days. The mean chla contents of cultured petioles (with or without kinetin) were not significantly different from those at day 0 after 3 days. For petioles cultured with kinetin the change in chla content between day 6 and 10 was significantly different at the 1% level, while that for petioles cultured without it was not.

The mean chlb content per petiole for petioles cultured with kinetin decreased between all the days measurements were taken. Again the same was true for petioles cultured without kinetin. The mean chlb contents for petioles cultured with kinetin were larger than those cultured without it on days 3 and 6. These differences were not significant at the 5% level. The decrease in the mean chlb content for cultured petioles relative to fresh petioles was significant at the 1% level after 3 days.

This experiment showed the following:-

(l) Petioles cultured with or without kinetin increased in fresh weight during culture.

(2) During culture top and bottom regions of petioles in contact with the growth medium swelled while the middle regions did not.

(3) Up to day 6 the increase in diameter of the top of petioles cultured with kinetin was significantly greater than those cultured without it. After 6 days the mean diameter of the bottoms were significantly larger for petioles cultured with kinetin.

(4) After l0 days there was no significant change in the mean length of petioles cultured with or without kinetin.

(5) There was no significant difference in the change in the chl concentration between petioles cultured with or without kinetin.

(6) The chl concentration in the cultured petioles decreased significantly betwen days 0 and 3 whether kinetin was present or not.

(7) The mean chla content of petioles cultured with kinetin decreased significantly slower than that for petioles cultured without it up to 3 days. After this time the opposite was true.

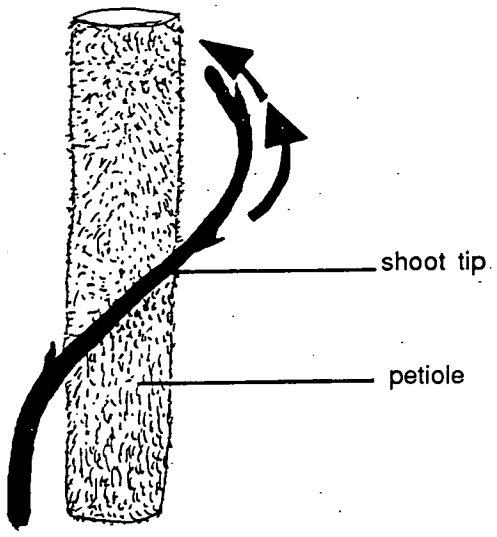
(8) The chl contents of the petioles decrease significantly with increasing time in culture beyond 3 days. The chlb contents of petioles cultured with or without kinetin decrease significantly between days 3 and 10. The same is true for the chla content of petioles cultured with kinetin. However the chla content of petioles cultured without kinetin does not fall significantly over this period.

Increasing the concentration of the kinetin to 0.4 mg l^{-1} did not reduce the changes in chl found in petioles supplied with 0.2 mg l^{-1}. Therefore the smaller concentration was used in all future experiments.

2.4 Interacting the host and parasite

Having determined appropriate culture conditions for both host and parasite the next step was to bring the two partners together.

Fig. 2.4a. During inoculation the concave side of the curved apical region of the *Cuscuta* shoot tip is placed next to the petiole so that the apex points anti-clockwise.



Single petioles were sterilised then prepared for culture in the manner described in sections 3.3 and 3.6 of Ch2. The petioles were cultured vertically in the centre of the plant container base as shown in Fig 2.3e. Four sterile 35mm Durham tubes were then pushed into the agar around the petiole at a distance of approximately l0mm. Sterile liquid *Cuscuta* medium (see section 3.6 Ch2) was pipetted into each tube. Then one sterilised 50mm *Cuscuta campestris* shoot tip was inoculated into each tube. The hook or curve of the apex of each shoot tip was carefully orientated in an anticlockwise fashion (see Fig 2.4a) at right angles against the vertical petiole. The containers were then transferred to a growth cabinet illuminated overhead by a single Cool Lite 40W fluorescent bulb.

Preliminary results. None of the shoot tips responded to their hosts by coiling. On the contrary all the shoot tips extended and grew towards the walls of the plant container. As previously mentioned the containers were made from a material which was an extremely poor electrical conductor and their surfaces became electrostatically charged. This caused the shoot tips to become attracted to the surfaces of the containers. This problem was overcome as described in section 2.3. In addition to this electrostatic interference it was suspected that the shoot tips were responding phototrophically to the single light source in the centre of the cabinet. An experiment was performed to see how the growing shoot tips of *Cuscuta campestris* responded to the lighting conditions in this growth cabinet. The experiment was performed with newly germinated *Cuscuta campestris* seeds. The advantage of using seeds was that their shoot tips had not yet emerged from the seed coat. All the directional growth which subsequently occurred could be interpreted as a result either of preprogrammed developmental processes or of response to the environment.

2.4.1 The effect of the direction of the light source on the direction of growth of germinating *Cuscuta campestris* apices

The aim of the experiment was to determine if *Cuscuta* seedlings grew towards the light source in the growth cabinet being used.

Experimental

Eighty seeds which had been simultaneously stimulated to germinate and sterilised were divided up and placed on a l% aqueous agar gel in the centre of five plant containers. The containers were sealed and arranged against the right-hand wall of the growth cabinet with the light source situated above and to the left. The seeds were then left undisturbed for one week. After this time each container was examined to see in which direction the shoot tips of the seedlings had grown. A line was drawn down the centre of the agar surface at 90^{o} to the direction of the incident light. The shoot tips were scored relative to whether they had grown to the right or to the left of this line. The results are shown in Table 2.4.la.

Table 2.4.Ia The numbers of shoot tips of germinated seedlings growing towards the light source one week after sterile seeds were sown inside plant containers.

Container Number	Seeds Germinated	Direction of Growth	
		L	R
1	6	4	2
2	8	7	1
3	8	5	3
4	9	7	2
5	6	5	1
Total	37	28	9

The direction of shoot tip growth

Out of a total of 37 shoot tips which germinated 28 grew towards the light source and 9 away from it suggesting that the growing tips were positively phototrophic. The fact that some shoot tips grew in the opposite direction suggests that the direction of the light source was not the only factor influencing the direction of growth.

After a result of this experiment all future interactions were performed in a growth cabinet with even illumination across the whole overhead width and length. At the same time an experiment was performed to demonstrate that the parasite shoot tip would coil under sterile conditions. The 'host' used in this experiment was not a petiole but a bamboo cane, chosen to circumvent the time-consuming preparations necessary to provide cultured host petioles. Fig 2.4c shows how the cultured parasite readily responded to the bamboo cane forming tight coils around it. Prehaustorial organs developed on the concave surface of the coils just as they did when on plant hosts. A transverse section through the *Cuscuta* stem coiled around the bamboo cane (see Fig 2.4d) shows how the prehaustoria developed haustoria which extended to the surface of the bamboo cane.

Having removed the obstacles of unidirectional light and electrostatic charge, an experiment was performed to determine the frequency of coiling of cultured parasite shoot tips when they were inoculated onto cultured *Pelargonium* petioles under the conditions being used.

2.4.2 The frequency of coiling *in vitro* when cultured parasite was inoculated on to cultured host

The aim of this experiment was to measure the frequency of coiling under the conditions being used.

Experimental

The plant containers were prepared with single petioles cultured in each container as described above in section 2.3 of this chapter. In four separate experiments parasite tips were inoculated onto the host as described above (previous section). The numbers of shoot tips in each experiment were 65, 26, 27 and 26 respectively. The containers were sealed with Parafilm and incubated in a growth cabinet with constant fluorescent illumination (120–170 μmoles $m^{-2}s^{-1}$ After 24 hr the frequency of coiling was recorded for each experiment. Shoot tips not coiling after this time were re-orientated under sterile conditions.

The frequency of coiling (Table 2.4.2a)

The frequencies of coiling obtained in each experiment were 13.8, 11.5, 7.4 and 11.5% respectively. These data showed that coiling was possible under the conditions being used, but at a low fequency. Figure 2.4.2b shows a host/parasite interaction *in vitro* after 14

Fig. 2.4c. A sterile shoot tip coiled around a bamboo cane (B) under sterile conditions. Prehaustoria are evident on the inside of the coiled region of the parasite. Scale = 10mm ▼

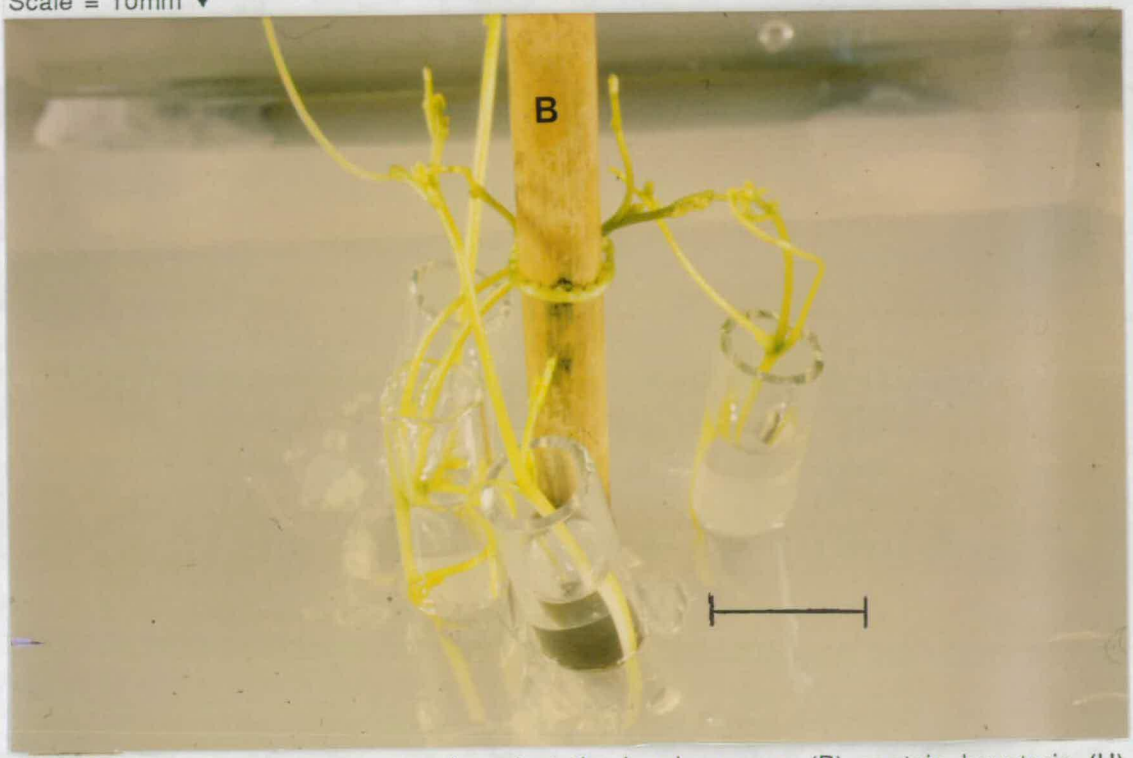


Fig. 2.4d. Prehaustoria appressed against the bamboo cane (B) contain haustoria (H). These haustoria are found to grow toward interface between the prehaustorium and the cane but not to penetrate the wood. Technical details: Light microscope, 0.5µm, resin embedded, Toluidine blue stained, magnification of X100. ▼

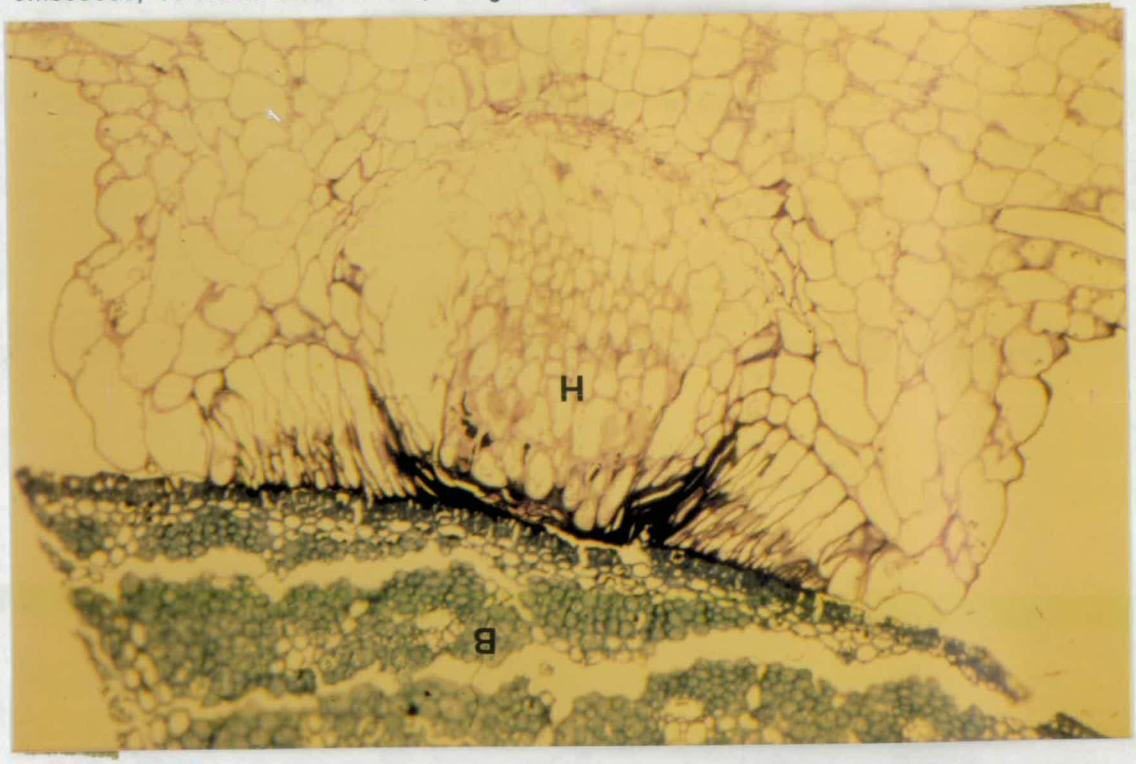
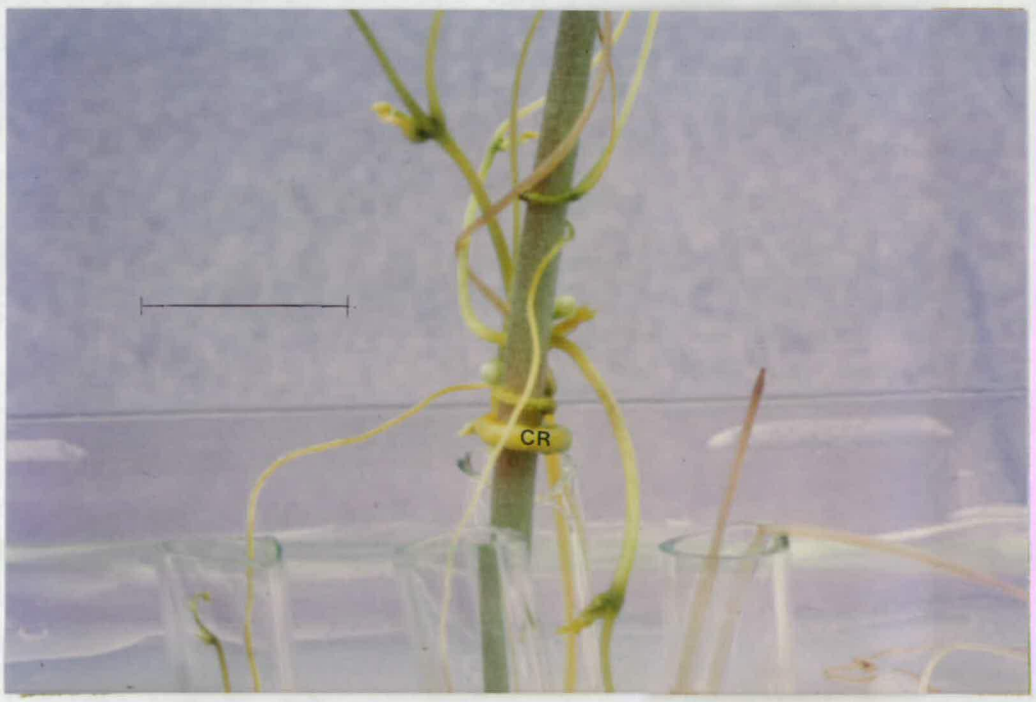


Table 2.4.2a The frequency of coiling when sterile *Pelargonium* petioles were inoculated with sterile cultured *Cuscuta campestris* shoot tips

Number of replicates	Number coiling	Percentage coiling
65	9	13.8
26	3	11.5
27	2	7.4
26	3	11.5

Fig. 2.4.2b. *Cuscuta campestris* shoot tips coiled around a *Pelargonium* petioles in a successful *in vitro* experiment. The coiled region of the parasite stem is swollen and the profuse growth indicates the tip advance stage has been reached. The host petiole is discoloured (red) and swollen in the vicinity of the coils. Scale = 10mm, (CR) = coiled region ▼



days. The coiled region has expanded relative to the rest of the shoot tip and the tip advance stage has been reached. Also evident is the swelling and discolouration of the petiole directly under the coiled region.

Other method of inoculation

This method of inoculating shoot tips *in vitro* was very time consuming with a low success rate. One of the problems was that it was difficult to secure the host and parasite together and keep them in contact. As a result other methods of inoculation were investigated. These methods were compared in order to determine which was the best.

2.4.3 A comparison of inoculation procedures for achieving infection *In vitro*

The aim of these experiments was to compare different methods of inoculation and find one which gave the highest frequency of coiling with the minimum of labour.

Cuscuta shoot tips 50mm long were used in all experiments. They were excised and sterilised as described in section 3.2 and 3.6 of Ch2. Some of the methods used did not involve immersing the shoot tips in growth media.

The different methods of inoculation that were used are as follows:-

(I) **The silicone rubber collar method**: This method was an extension of the method described in section 2.4.2. The hook or curve of each cultured shoot tip was orientated in an anti-clockwise manner against the vertical cultured petiole (Fig 2.4.b). In addition to this the shoot tips were held in position by applying sterile silicone rubber collars via sterile forceps (Fig 2.4.3a). Collars were made by cutting circles from lengths of tubing.

(2) **The glass capillary method:** The cut ends of sterilised 50mm *Cuscuta* shoot tips were fed down 30mm long glass capillaries with sterile forceps (see Fig 2.4.3b). The glass capillaries containing the shoot tips were pushed into the agar in the base of the plant container adjacent to the petiole. The apex was then orientated against the petiole at right angles in the usual way (see section 2.4.2). This method enabled the shoot tip to be firmly held against the petiole.

(3) **The simple alignment method:** Sterilised 50mm shoot tips were laid on the surface of the agar at right angles to the vertical petiole (see Fig 2.4.3c). The concave side of the curved apex was wrapped around the petiole in an anti-clockwise manner.

One experiment was performed with the silicone rubber collar method using 20 parasite shoot tips. Two experiments were performed using the glass capillary method inoculating 20 and 14 tips in each experiment respectively. Four experiments were performed using the simple alignment method. The number of tips inoculated in each was 30, 24, 40 and 40 respectively.

Fig. 2.4.3a. The silicone rubber collar method involves cultured parasite shoot tips being inoculated onto cultured host petioles as described in Fig 2.4b. In addition to this a silicone rubber collar is fastened around the host and parasite to hold the two together. Collars are made from split rings cut from silicone rubber tubing. They are applied via curved forceps.

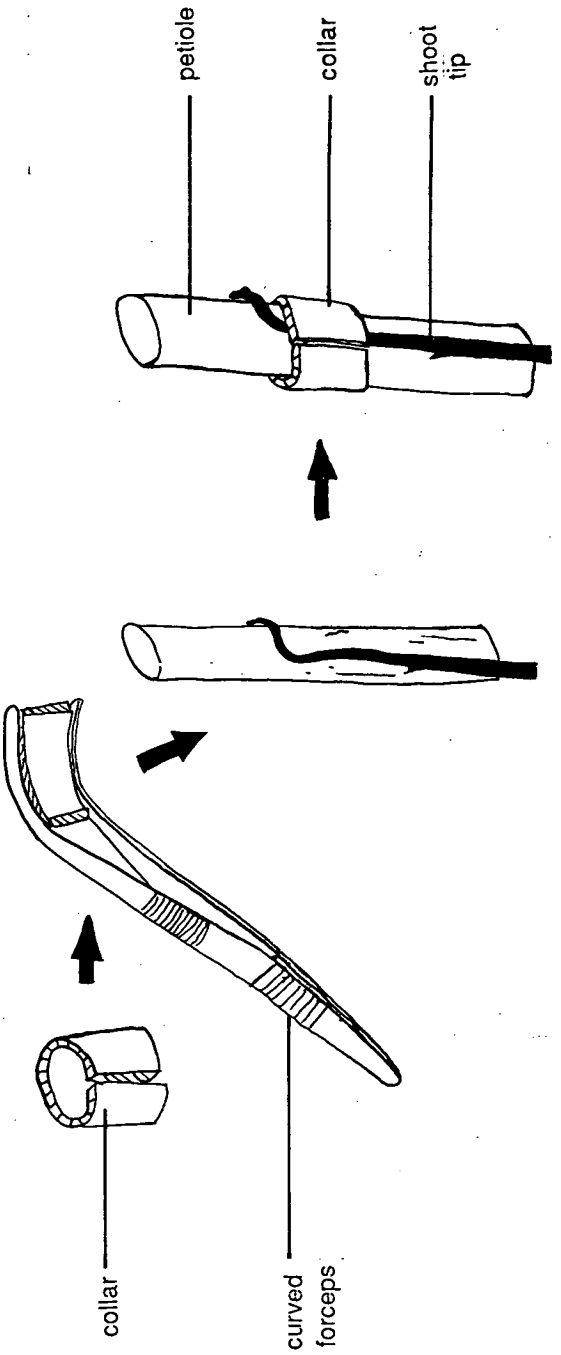


Fig. 2.4.3b. In the glass capillary method, sterile parasite shoot tips are carefully fed down short glass capillary tubes leaving the curved apical region protruding. Capillary and shoot tip are then orientated against the cultured host. The orientation of the apical region of the shoot tip is as illustrated in Fig 2.4b. The capillary tube acts as a rigid support holding the shoot tip in contact with the host.

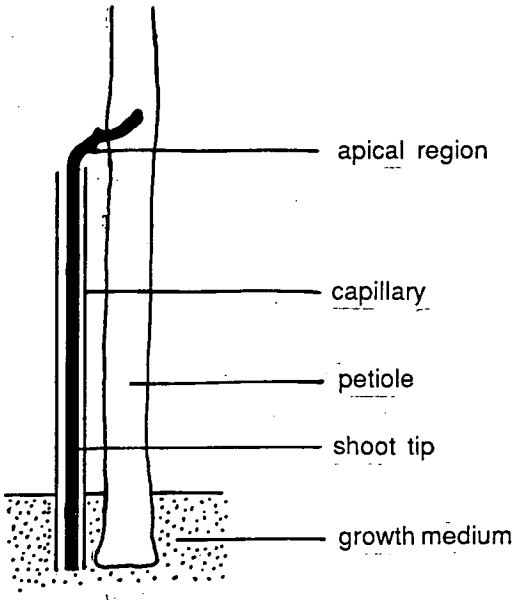
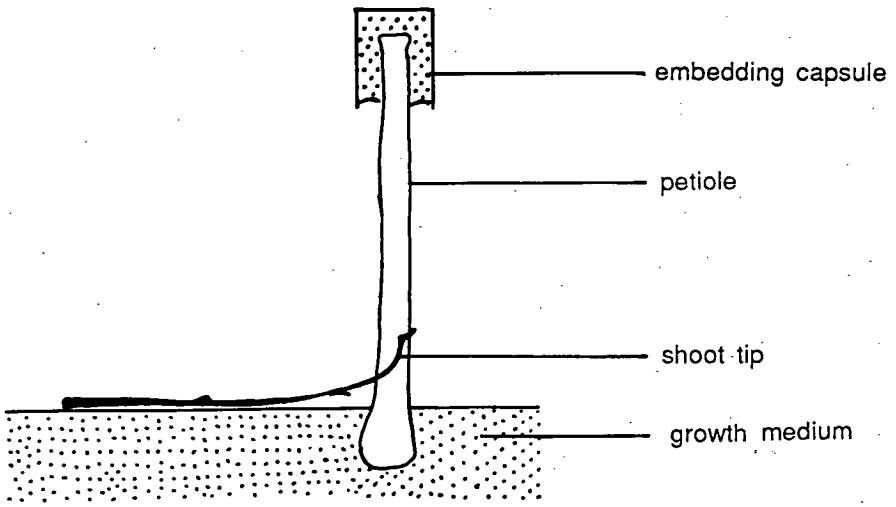


Fig. 2.4.3c. The simple alignment method involves excised, sterilised shoot tips being laid horizontally across the surface of the growth medium. The apical region is orientated against the cultured petiole as illustrated in Fig 2.4b.



The frequency of coiling (Table 2.4.3d)

None of the shoot tips which were inoculated using the silicone rubber collars coiled around their hosts. Ten percent of the shoot tips inoculated via glass capillaries coiled round their hosts in the first experiment and fifteen in the second. When shoot tips were inoculated by the simple alignment method, coiling occurred in 10, 16.7, 7.5 and 17.5% of cases respectively.

Summary of findings

The experiment showed that the simple alignment method produced the highest frequency of coiling (15.8%) followed by the glass capillary method (12%). The silicone rubber collar method resulted in no coiling at all.

The most suitable method of inoculation

Three methods used to inoculate *Cuscuta* shoot tips on to the host gave similar frequencies of coiling, the cultured host and parasite method (section 2.4.2) the simple alignment method (section 2.4.3) and the glass capillary method (section 2.4.3). Two of these methods involve sterile but non-cultured shoot tips. The advantages of using cultured shoot tips have been discussed already. However, their use added significantly to the time required to set up the system. In order to minimise the time and labour involved in setting up it was decided to use non-cultured unit lengths of clonally propagated shoot tips as the routine infective agents. The cultured shoot tip could still be used when necessary, for example to label the parasite or during attempts at chemical manipulation.

In order to increase the probability of coiling four shoot tips were inoculated per cultured host. It was found that in the majority of cases if coiling occurred *in vitro* all the subsequent stages of infection were also achieved.

A routine method for interacting host and parasite

The routine procedure for interacting host and parasite *in vitro* is summarised in Fig 2.4e. It shows, beginning at the top, each stage that the host and parasite moved through from initial excision to the interaction with each other.

Having developed the system to this stage it was now necessary to demonstrate that the important events (such as penetration and vascularisation) which occurred in *in vivo* interactions, also occurred during *in vitro* interactions. This would confirm that the *in vitro* system was representative of the *in vivo* interaction.

The next section describes the structural and ultrastructural changes which take place during an interaction between *Cuscuta campestris* shoot tips and *Pelargonium* petioles *in vitro.*

Table 2.4.3d The frequencies of coiling shown by sterile *Cuscuta campestris* shoot tips which were inoculated using four different methods onto cultured petioles

method	frequency of coiling (%)
glass capillary	10 15
simple alignment	10, 16.7 17.5 7.5
cultured parasite	13.8, 11.5 11.5, 7.4
silicone rubber collar	00

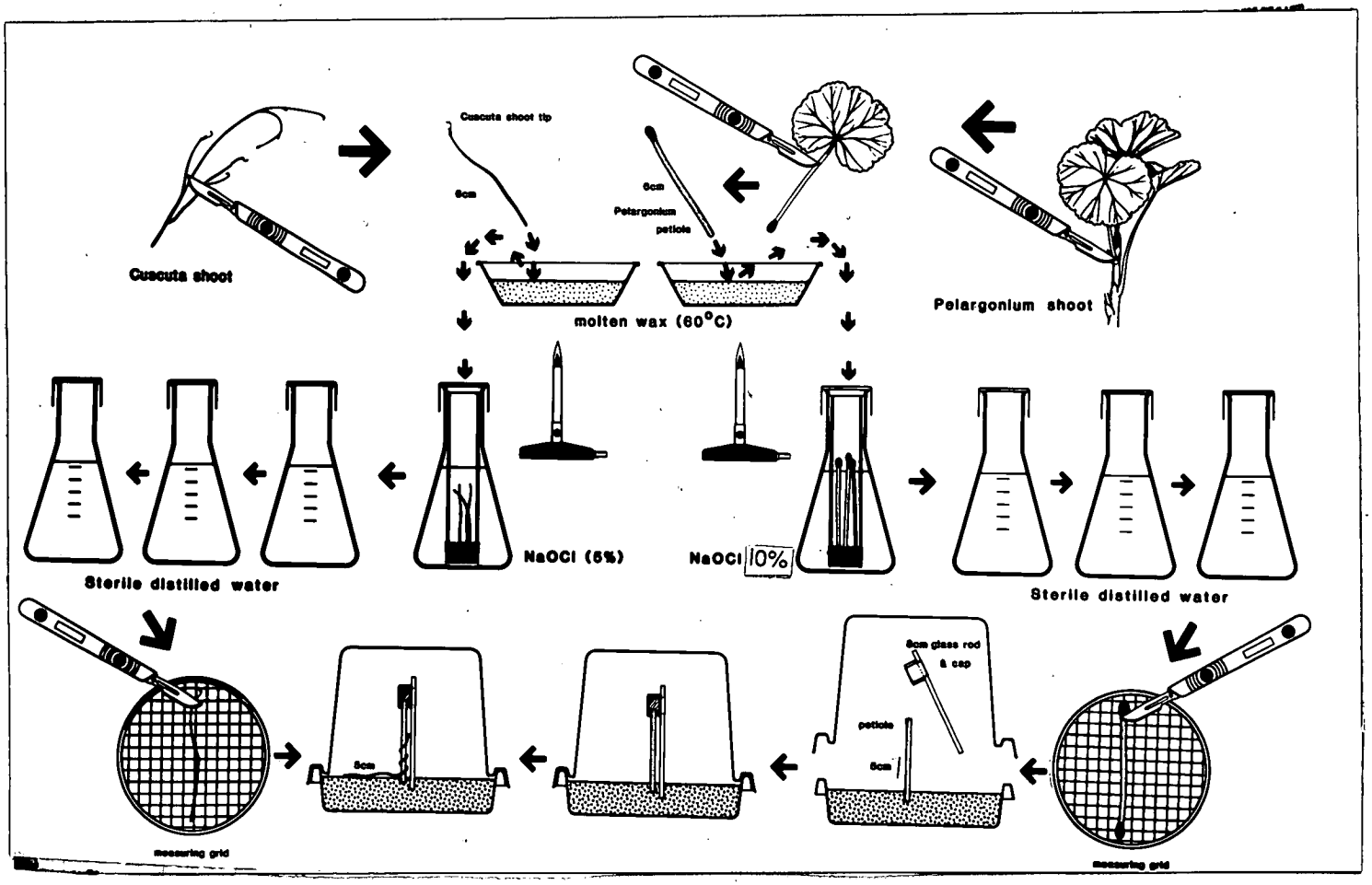


Fig. 2.4e. An illustrated summary of all the stages involved in the assembly of an *in vitro* host/parasite interaction. The process begins at the top of the diagram with the excision of the plant material and is complete when a sterile shoot tip coils around a sterile cultured host petiole.

2.5.The structural and ultrastructural events which occur during the infection of *Pelargonium* petioles by *Cuscuta campestris* shoot tips *in vitro*

The aim of this experiment was to demonstrate that the major events occurring during the invasion of *Pelargonium* petioles by *Cuscuta in vivo* also occur *in vitro*

Experimental

A population of host/parasite interactions were set up as described in section 2.4. Plant containers were incubated in the growth cabinet under constant illumination (see section 2.4). After 24hr each container was observed to see if any of the *Cuscuta* shoot tips had coiled around the host petiole. Those which had not coiled were re-orientated under sterile conditions. Three host/parasite interactions were harvested I, 3, 4, 5, 7, I0 and I4 days after inoculation. Each interaction was fixed, dehydrated and embedded as described in section 4.I.I of Ch2. Resin-embedded sections were cut and stained for light and transmission electron microscopy, as described in section of 4.3 Ch2. Ultrathin sections were cut from a resin block containing a 4 day-old *in vitro* host/parasite interaction. These sections were stained with lead/uranyl acetate (see section 4.3 of Ch2) and viewed with the Transmission Electron Microscope.

Observation of *In vitro* Interactions

Fig. 2.5.a shows an *in vitro* interaction harvested 24hr after inoculation and prepared for light microscopy. The coiled parasite shows deeper staining on the inner region closest to the host as seen in parasites in *in vivo* interactions at a similar stage. The distance between the host and parasite is not representative of the real distance after 24hr of parasitism. The separation was caused during specimen preparation. Fig 2.5.b shows an *in vitro* interaction in which penetration of the cultured host has taken place. The initial penetration has been completed, the sclerenchyma (sc) layer has been penetrated and search hyphae (sh) can be seen pushing between and into host cells. Search hyphae shl and sh2 can be seen to be pushing into host parenchyma cells. Some of the cells in this section are discoloured (dcl.dc2). Fig 2.5.c is a section of an *in vitro* interaction at a similar stage to that in the previous figure. However in this section the search hyphae have contacted the host vascular bundle (vb). At either side of the base of the haustorium inside the prehaustorium are areas of small darkly- staining cells. These cells appear similar to the early stages of haustorial development seen in Fig I.2.g of section I, and may be secondary haustoria. The host in this section does not show any areas of unusual division but some of the cells are discoloured (dcl, dc2).

Fig. 2.5.a. A transverse section through part of the coiled region of a sterile *Cuscuta campestris* (P) shoot tip infecting a cultured *Pelargonium* (H) peticles 24hr after inoculation. This section shows the contrast between the density of the cytoplasm of cells on the concave (IR) and convex sides of the coil. Technical details : Light microscope, 0.5μm thick, resin embedded, Toluidine blue stained, magnification X 95 .

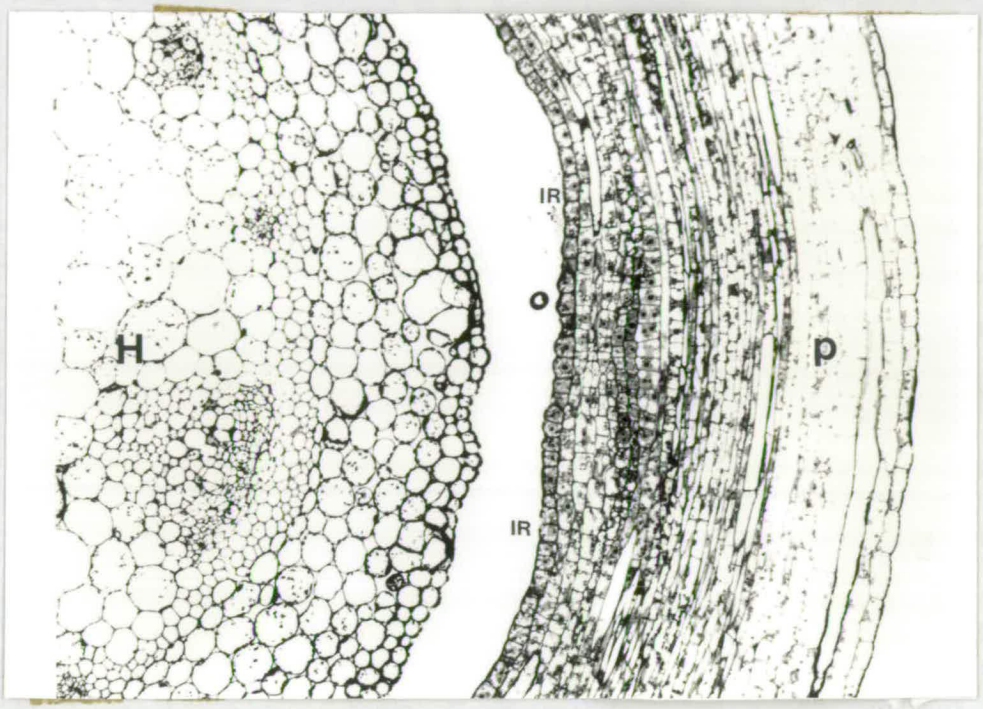


Fig. 2.5.b. A transverse section through part of the coiled region of a sterile *Cuscuta campestris* shoot tip (P) infecting a cultured *Pelargonium* (H) petiole when penetration has taken place (approximately 4 days after inoculation). This section shows search hyphae moving through the host cortex having traversed the sclerenchyma (sc). The search hyphae (sh1 and sh2) appear to be entering host cells. Discoloured cells (dc1 and 2) Technical details : Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification X 95 .

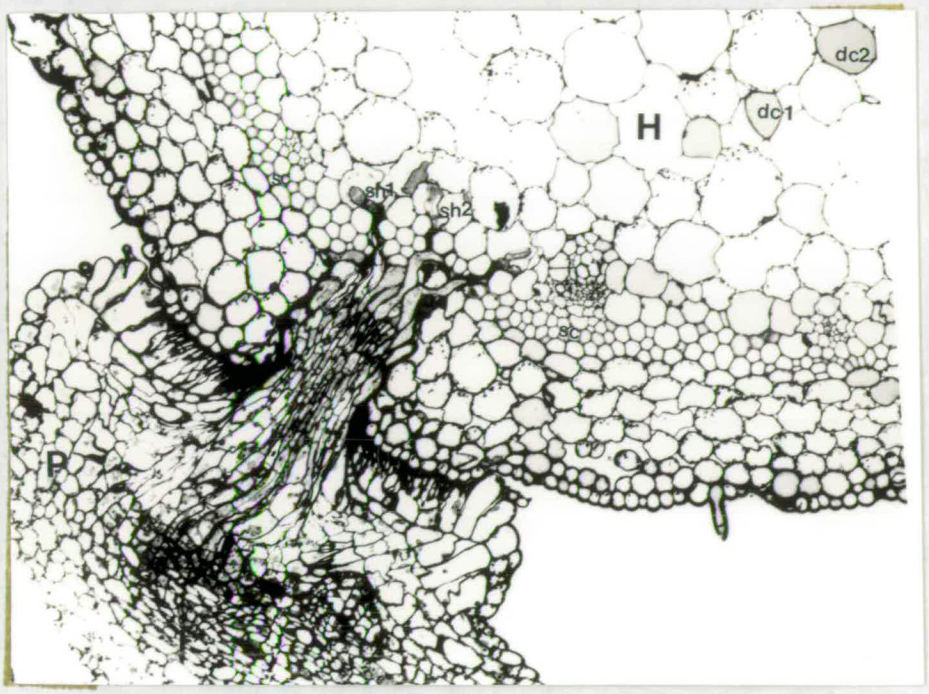


Fig. 2.5.c A transverse section through part of the coiled region of a sterile *Cuscuta campestris* shoot tip (P) infecting a sterile cultured *Pelargonium* (H) petiole when contact with a vascular bundle (vb) has been made. This section shows the search hyphae of both haustoria (ha) growing towards the vascular bundle. Those of ha2 have made contact with this vascular bundle. This section shows dark stained groups of cells at the base of haustoria which resemble haustrial initials (hai). (dc1 and dc2) = discoloured cells. Technical details : Ligh microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification X 76

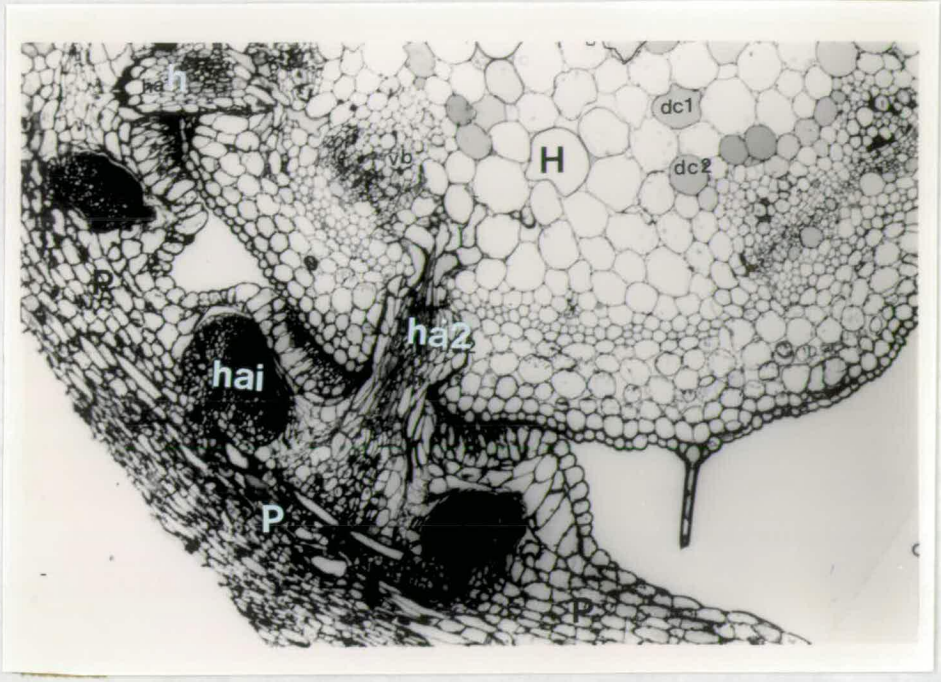

ha1
vb
H
dc1
dc2
hai
ha2
P
P

Fig 2.5.d shows another *in vitro* interaction. The most striking feature of this interaction is the cell division which has occurred in the outer parenchyma cells of the petiole. The dividing regions (dvl and dv2) are not in direct contact with the parasite but are adjacent to it. The dark-stained cells seen in other *in vitro* interactions at the base of the haustorium are also seen in this one.

Fig 2.5e shows an *in vitro* interaction at a later stage. One of the haustoria (hal) has made contact with a host vascular bundle (vbl). The search hyphae (sh) have pushed into the centre of the host vascular bundle. The central cells of the haustorium have become secondarily thickened. This is the usual way in which a vascular bridge is formed between the host and parasite. The host cells in the central region of the petiole at C appear to have undergone some secondary division. An irregular pattern of cross walls has been laid down within the usually regular hexagonal shaped parenchyma. The parasite cells are not in direct contact with these dividing host cells. This is assuming that search hyphae from a haustorium in another plane have not penetrated this far into the petiole.

TEM sections of penetrating search hyphae

The region at sh in Fig 2.5.b where the parasite search hyphae appeared to be entering the host cells has been enlarged in Fig 2.5f. A search hypha is shown apparently pushing into a host parenchyma cell at pe. This region was isolated on the resin block-face forming a meser. Thin sections were cut from the face of the meser and stained with lead/uranyl acetate, than viewed with a Transmission Electron Microscope. Fig 2.5g is a composite of two pictures from region pe in Fig 2.5f. The host and parasite are labelled H and P respectively. The major part of the host cell which was penetrated was vacuole (V). Within the vacuole there were several unidentified vesicular structures. In the region cv there appeared to be a break in the tonoplast with vacuolar contents and cytoplasmic organelles mixed together. The majority of the cytoplasm of the host appears as a narrow granular band along the cell wall (hcw). The host and the parasite walls (pcw) are different thicknesses at different points along the interface seen here. The region E represents the entry point of the parasite into the host cell. In this region the host cell wall is particularly thick. The host wall is at its narrowest along the region where the parasite has pushed into the cell. Within the interfaced walls of the host and parasite there is some evidence of structures resembling plasmodesmata (see pdl, pd2, pd3 and pd4). In pdl-3 all the structures are confined to the parasite wall. At pd4 the structures are seen in both walls but they do not extend all the way across, at least when viewed in the plane of this section.

Fig 2.5.h shows an enlargement of the contact point between the host and the parasite at the entry point into the host cell. The plasmodesmatal structure pd2 extends all the way across the parasite wall ending in a larger rounded structure. The structure pdl does not

Fig. 2.5.d A transverse section through part of the coiled region of a sterile *Cuscuta campestris* shoot tip (P) infecting a sterile, cultured *Pelargonium* (H) petiole showing host cell division This section shows radial cell division in the outer parenchymatous region at DV 1 and 2. Cell division has occurred in cells not directly on contact with the penetrating haustroium. Technical details : Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification X 76

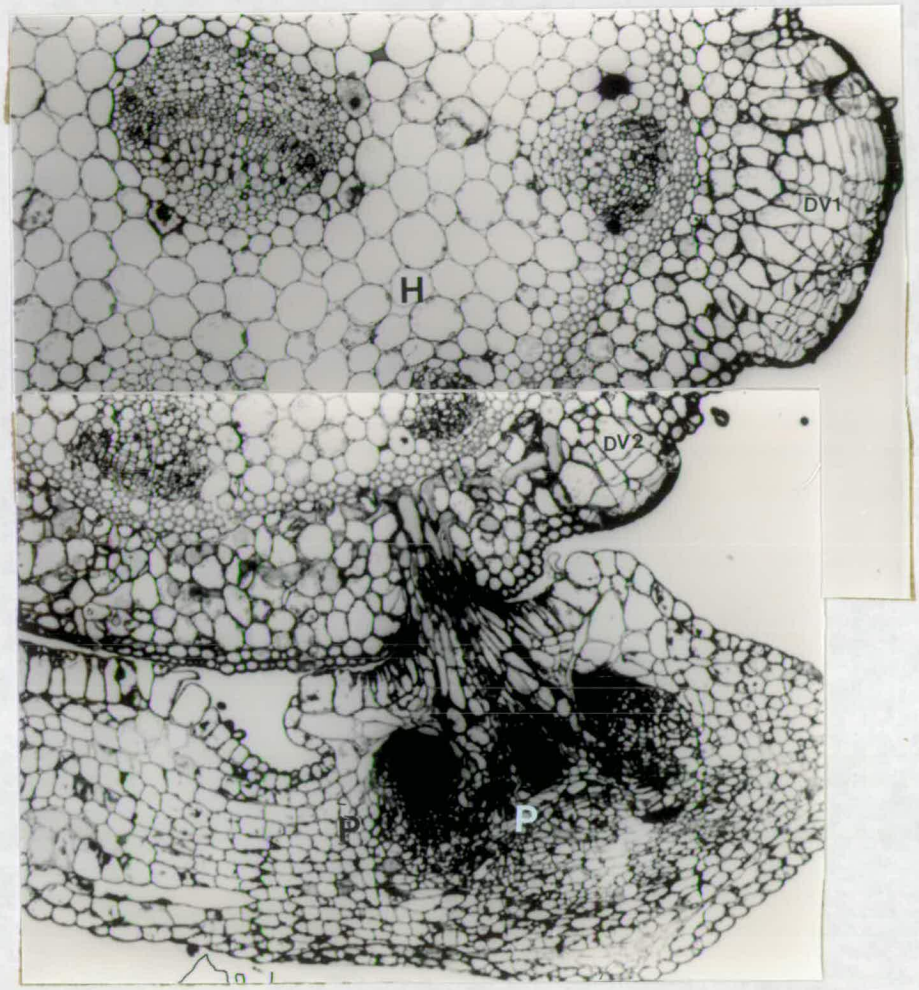


Fig.2.5e A transverse section through part of the coiled region of a sterile *Cuscuta campestris* shoot tip (P) infecting a sterile cultured *Pelargonium* (H) petiole showing the development of a vascular channel. This section shows that search hyphae from the haustorium (ha1) have contacted the vascular bundle (vb1). The cells in the centre of the haustorium and search hyphae (sh) are becoming lignified. There is also some disorganised cell division in the central region (C) of the host cortex of the host petiole. Technical details : Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification x ×95 ▼

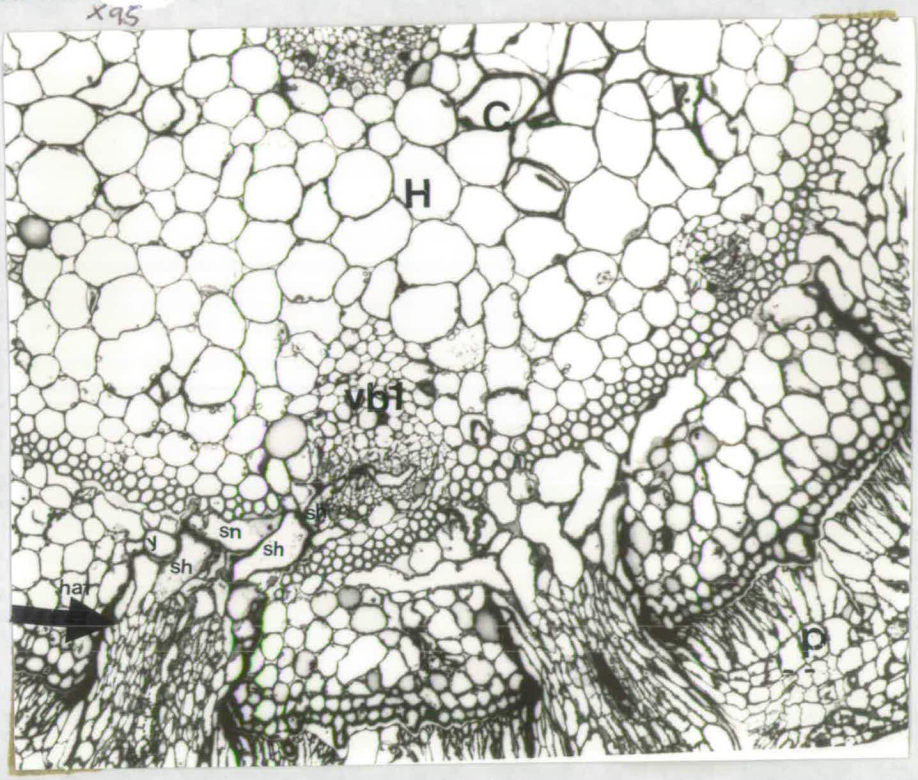


Fig. 2.5.f A small part of a transverse section through the coiled region of a sterile *Cuscuta campestris* shoot tip (P) infecting a sterile cultured *Pelargonium* (H) petiole showing the advance of the search hyphae (sh) through the cortex. The search hyphae have traversed the host sclerenchyma (sc) layer by moving along the middle lamellae. In contrast in the cortex search hyphae appear to be actively penetrating host cells.(pe) = penetration point. Technical details: Light microscope, 0.5µm thick, resin embedded, Toluidine blue stained, magnification x 570.

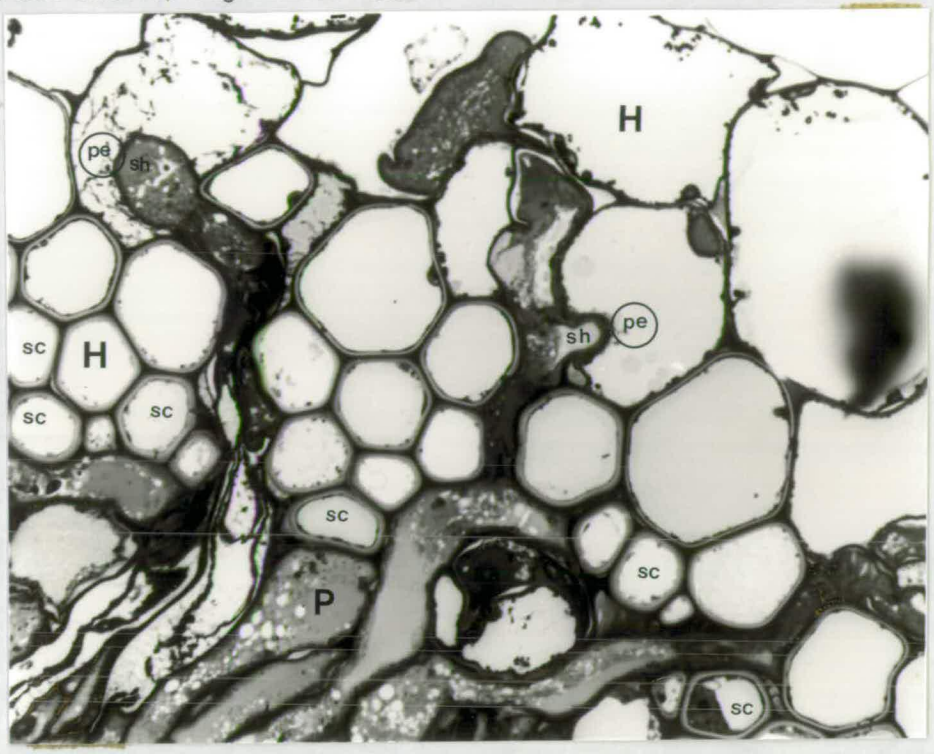


Fig. 2.5.g A TEM micrograph showing part of a *Cuscuta campestris* (P) search hypha penetrating a host cell (H). The host cell is mainly vacuole (V) with a band of cytoplasm (CT) below the cell membrane and wall. A host and parasite wall (hcw and pcw) are evident all along the length of the protruding structure. At the point of entry at E the wall of the host is much thicker than at other points. Adjacent to this there appears to be a mixing of host cytoplasm and vacuole contents (cv). In the parasite wall at pd 1, 2, 3 and 4 there appears to be some plasmodesmatal like structures. Technical details : Lead citrate/Uranylacetate stained, magnification x 5700 SA

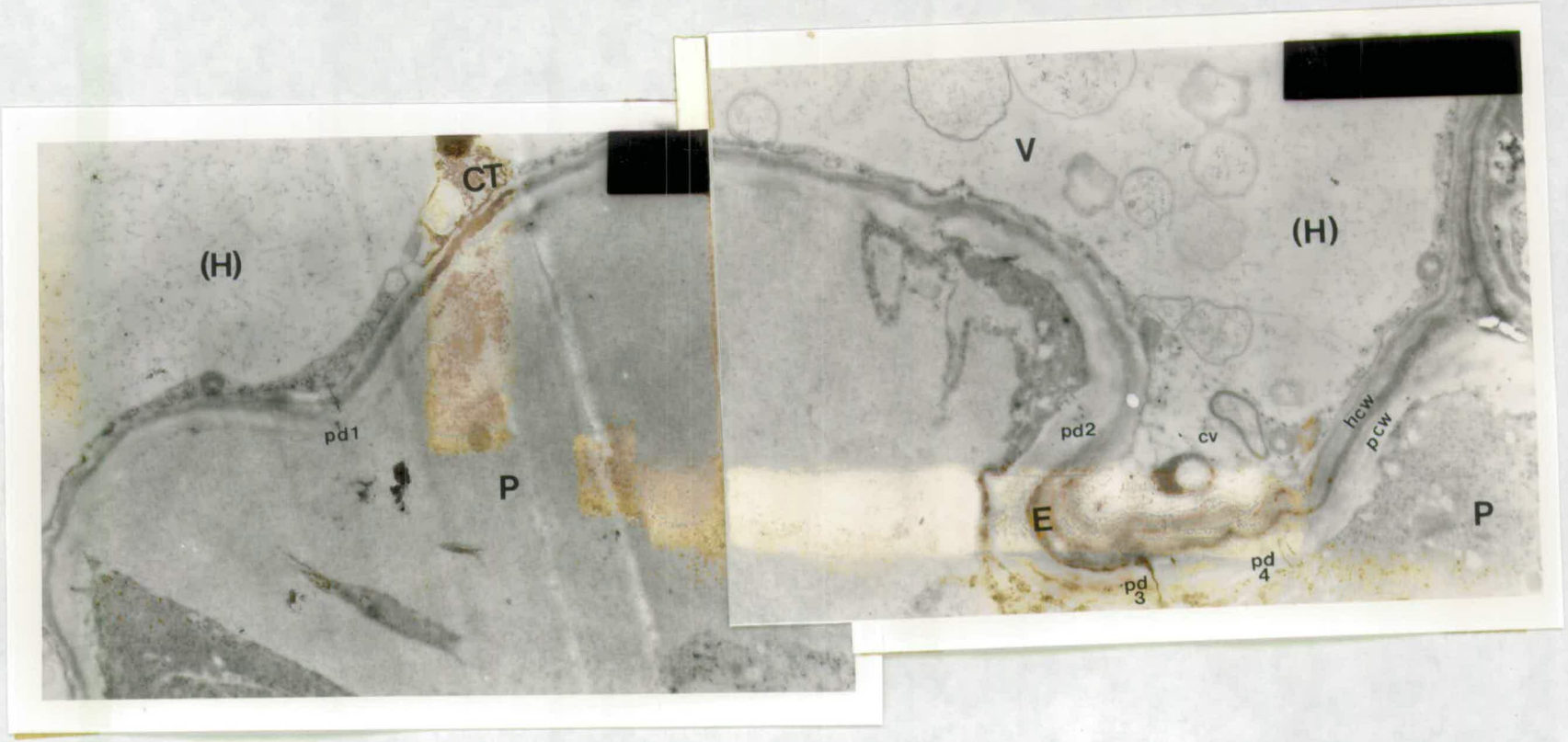
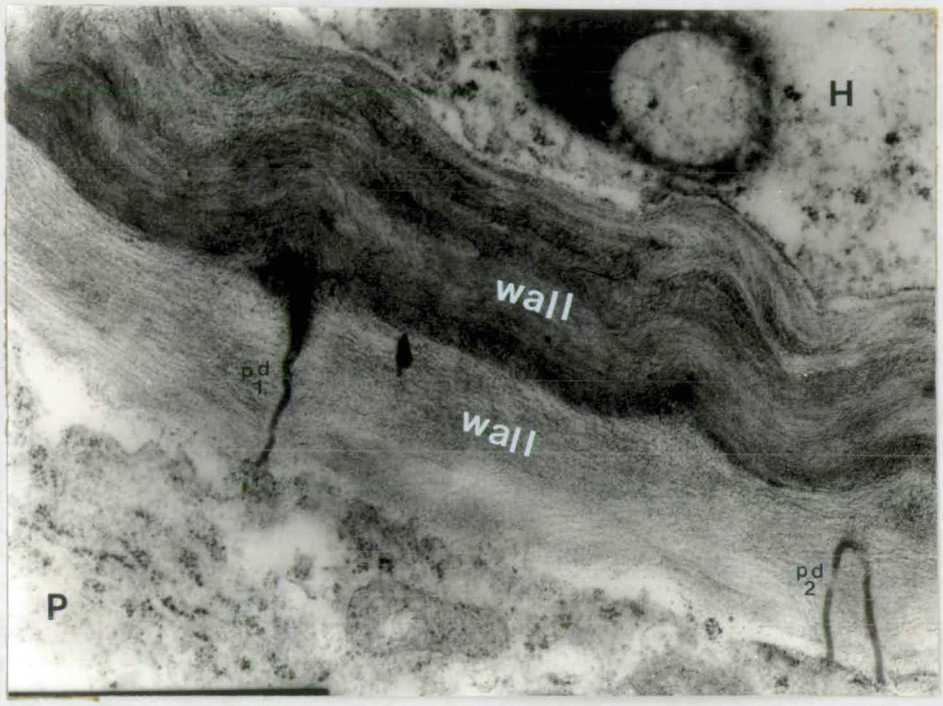


Fig. 2.5.h A TEM micrograph showing the close proximity of the walls of *Cuscuta campestris* (P) and *Pelargonium zonale* (H) adjacent to the point of entry of the parasite into a host cell (see Fig 2.5.lh). The parasite wall contains plasmodesmatal type structures (pdl, pd2) one of which extends across the whole of its width. There are no corresponding structures in the host wall. There is no visible intervening layer between the host and parasite cell walls. There are some differences between the two walls shown by their different staining properties. Technical details : Lead citrate/Uranylacetate stained, magnification x ×22,125



completely traverse the wall in this section. This figure shows that host and parasite walls are in intimate contact, evidently without an intervening layer between the two organisms.

A Summary of the major points arising from this experiment

The experiment showed the following:-

(I) Sterile coiled *Cuscuta* shoot tips develop haustoria and penetrate cultured *Pelargonium* petioles.

(2) The haustoria develop search hyphae which are able to penetrate the sclerenchyma layer, subsequently making contact with host vascular bundles and forming vascular channels to connect the parasite to the host.

(3) TEM micrographs show a search hyphae penetrating a host parenchyma cell. The host wall invaginated and the parasite pushed into it. The host cell-wall was thickest at the entry point of the parasite and thinnest at the advancing interface.

(4) Structures resembling plasmodesmata were found in the walls of a parasite cell in intimate contact with a host cell. These structures in some cases traversed the whole of the parasite wall but did not continue into the adjacent host wall.

(5) In most cross sections a number of the host cells were discoloured.

(6) Groups of densely-cytoplasmic cells were found at the base of penetrating haustoria which resemble immature haustoria.

(7) Several transverse sections of invaded petioles revealed division not observed in any of the *in vivo* interactions. Divisions were observed in both the outer and inner layers of parenchyma. They were not associated with direct search hyphae contact.

(8) No intervening layer was found between host and parasite cell walls even when the parasite cell was physically inside the host cell.

All the major events which occur during invasion of *Pelargonium* petioles by *Cuscuta* shoot tips *in vivo* were observed in the *in vitro* interactions. There were more cases of unusual cell divisions in the in *vitro* material looked at than had been found in the *in vivo* material.

Summary of Section 2

This section has described the development of a*n in vitro* system for the study of cellular interactions between *Pelargonium zonale* and *Cuscuta campestris*. The development of the system involved the following:-

(I) Assessment of the relationship between ability to infect and inocula length.

(2) Assessment of the length of time shoot tips retain their ability to infect following excision.

(3) Assessment of the impact of sterilisation and culturing on the infectivity of excised shoot tips.

(4) Culture of host and parasite.

(5) Establishment of a suitable cultural environment for coiling to occur.

(6) Recording of structural and ultrastructural changes which occurred during parasitism *in vitro* and comparing these to the *in vivo* changes.

The structural examination *in vitro* and *in vivo* provide valuable information concerning the mechanism of host invasion by the parasite. The next section describes an investigation into the role of cell wall degrading enzymes in this process.

Section 3 An investigation into the role of certain cell wall degrading enzymes in the infection of *Pelargonium* petioles by *Cuscuta campestris* shoot tips

Cell wall degrading enzymes have long been thought to have a role in the penetration of host plants infected with *Cuscuta* (Thomson 1925)

In this section a study has been made of three types of cell wall degrading enzymes: cellulase (CE), polygalacturonase (PGURASE) and pectin esterase (PE). Particular attention is paid to the patterns of activity displayed by each enzyme in the host and the parasite during penetration and establishment of *Cuscuta* on *Pelargonium* petioles. The results for each type of enzyme are described separately.

3.1 Cellulase activity in the host and parasite

Attention was first focussed on the parasite.

3.1.1 A Comparison of the cellulase activity present in extracts of coiled and uncoiled stems of *Cuscuta campestris*

The aim of this experiment was to measure the CE activity in coiled, penetrating and uncoiled *Cuscuta campestris* stems in order to see if there were any differences in this respect between the two parts of the plant.

Experimental

Plant material used in the assays was taken from greenhouse stock plants. The uncoiled stem tissues were excised with a sharp razor blade from young and mature internodes. Material was cut at least 10mm from the apex or from any nodes. Individual tight coiled regions of stem which bore penetrating haustoria were carefully dissected away from the *Pelargonium* petioles on which they were growing . The extraction procedure performed on coiled and uncoiled tissues was exactly the same. Protein was extracted from five 0.1g samples of coiled stem, and five from uncoiled stem by the freeze/thaw method (see section 5.1.2 of Ch2). Using the viscometric method, CE assays were performed on 0.1ml aliquots of each protein sample at 21^{o}C in a constant temperature room. Flow times were recorded at 0, 0.5, 1.0 and 1.5 or 3.0 hr (1.5 for coiled and 3.0 for uncoiled). Extraction buffer mixed with the substrate in the same proportions and under the same conditions as extracts and substrate served as controls.

The amounts of protein present in each extract were estimated by assaying three, 30µl aliquots of each protein sample by Beardens method (see section 5.3.1 of Ch2). The enzyme activities were calculated for each sample and expressed per ml of extract and per mg protein (Table 3.1.1a). Means, standard deviations and standard errors were calculated for the protein contents and CE activities for each type of tissue (Table 3.1.1b). The significance of the variability in enzyme activity and protein content between extracts

Table 3.I.Ia The cellulase (CE) activity in the extract of coiled (C) and uncoiled (U) parts of *Cuscuta campestris* stems parasitising *Pelargonium* petioles. (Activity expressed in units per mg protein and per ml of extract per hr).

Sample number	Type of extract	Hours after start of assay	Activity per ml	Activity per mg protein
1	U	0.5	0.820	0.656
		1.0	1.200	0.960
		3.0	1.077	0.861
2	U	1.0	0.340	0.294
		3.0	0.453	0.391
3	U	0.5	1.675	0.989
		1.0	2.040	1.204
		3.0	1.180	0.697
4	U	0.5	0.700	0.537
		1.0	1.300	0.997
		3.0	1.303	0.999
5	U	0.5	1.860	1.412
		1.0	1.980	1.503
		3.0	1.380	1.048
6	C	0.5	5.680	2.444
		1.0	4.680	2.014
		1.5	4.167	1.793
7	C	0.5	12.380	5.427
		1.0	12.450	5.458
		1.5	12.893	5.652

Contd.

Sample number	Type of extract	Hours after start of assay	Activity per ml	Activity per mg protein
8	C	0.5	13.080	5.230
		1.0	12.000	4.798
		1.5	10.540	4.214
9	C	0.5	5.300	2.714
		1.0	5.080	2.601
		1.5	6.130	3.139
10	C	0.5	8.800	4.243
		1.0	8.250	3.978
		1.5	6.720	3.240

Table 3.1.1b The means, standard deviations (s.d.) and standard errors (s.e.) of the cellulase (CE) activities in coiled and uncoiled *Cuscuta* stem parasitising *Pelargonium* petioles.

(1) Per ml extract

type of extract	Time (hrs)	mean	s.d.	s.e.
U	0.5	1.264	± 0.589	± 0.294
	1.0	1.372	± 0.692	± 0.309
	3.0	1.079	± 0.368	± 0.165
C	0.5	9.048	± 3.634	± 1.625
	1.0	7.692	± 4.855	± 2.171
	1.5	8.089	± 3.541	± 1.584

(2) Per mg protein

type of extract	Time (hrs)	mean	s.d.	s.e.
U	0.5	0.898	± 0.392	± 0.196
	1.0	0.992	± 0.446	± 0.199
	3.0	0.799	± 0.265	± 0.119
C	0.5	4.012	± 1.386	± 0.620
	1.0	3.770	± 1.449	± 0.648
	1.5	3.265	± 1.455	± 0.651

of different types of tissues was determined by using analysis of variance.

Results

The amounts of protein in extracts made from samples of uncoiled *Cuscuta* stem ranged from 1.16 to 1.69 mg per ml while amounts in coiled stem from 1.95 to 2.50 mg per ml (see Table 3.1.1a). The amounts of protein extracted from samples of coiled *Cuscuta* stem were significantly greater than amounts from uncoiled stem at the 5% level. All the values of CE activity per ml of extract obtained for extracts of coiled stems (Table 3.1.1a) were greater than those for extracts of uncoiled stems. The same was true when the activity was expressed on a per mg protein basis (Table 3.1.1a). When the mean CE activities per ml and per mg protein of extracts of coiled stems after 0.5 and 1.0 hr of incubation (Table 3.1.1a) were compared to the corresponding values for extracts of uncoiled stems (Table 3.1.1a), significant differences were found at the 5% level. The significantly greater CE activity per mg protein in coiled stems compared to uncoiled stems demonstrated that the larger CE activity in coiled stems was not just a result of an overall increase in the protein concentration.

This experiment showed that both the protein concentration and the activity of coiled *Cuscuta* stems were significantly greater than those in uncoiled stems.

A higher CE activity in the haustorial region of the parasite may indicate that this type of enzyme is involved in the penetration process. If this were the case it might be expected that increases in the CE activity of tissues in close contact with the host would to some extent coincide with the physical penetration of it. An experiment was therefore performed to determine how the CE activity within the tight coiled region changed with time during invasion of the host. This experiment is described below.

3.1.2 The change in cellulase (CE) activity in extracts of *Cuscuta campestris* tight coiled regions from 2-11 days after initial coiling

The aim of this experiment was to determine how the CE activity of the tight coiled region changed with increasing age during the invasion of *Pelargonium* petioles by *Cuscuta campestris.*

Approximately two hundred 50mm shoot tips of *Cuscuta campestris* were inoculated on to petioles of expanding leaves of *Pelargonium* plants in the manner described in section 2.2.2 of Ch2. It took 2 days to complete and number all the inoculations. Following inoculation each shoot tip was checked daily to see if it had coiled around the petiole. Every day for 2-11 days after the start of the experiment, tight coiled regions were harvested by dissection from excised petioles in the cold room and weighed on a Sartorius digital balance. Protein was extracted from four, 80 mg samples of coiled regions by the freeze/thaw method described in section 5.1.2 of Ch2. The first freeze step was extended

from 2.5 to 5.5 hr. The number of coiled regions per sample was recorded. Aliquots of crude extract were used in viscometric CE assays and Bearden protein assays. Reaction mixtures for the CE assays consisted of 25µl crude extract mixed with 75µl of extraction buffer and 200µl of 1% carboxy methyl cellulose. In controls the 25µl crude extract was substitued with 25µl of boiled crude extract. Four different crude extracts were used in the assays on each day except for day 2 and 6 when 5 extracts were used. The CE activity was calculated per ml of extract, per mg protein and per coil.* Having calculated the mean values and their standard errors and standard deviations the mean activities were plotted against time.(*–After 2.5hr)

The change in the number of coiled regions per sample

In section I it was found that coiling occurred within 24hr of inoculation and thereafter the length of the coiled region did not increase. In this experiment the number of coiled regions per 80mg samples decreased with increasing age of the interaction (Table 3.I.2). The mean fresh weight per unit length of the coiled regions must therefore have increased with time.

The change in the protein content of the tight coiled regions

The change in the mean protein concentration of crude extracts with time can be seen in Fig 3.I.2a. The mean protein content of the crude extracts decreased between 2 and 3 days after inoculation. There was a further fall between 3 and 4 days. The mean value then remained constant between 4 and 5 days before a slight decrease between 5 and 6 days. For the next 2 days there was a fall in the mean protein content of the crude extracts. Thereafter there were moderate increases in the mean content each day from 8 to II days. When the protein concentrations in crude extract of different ages were compared using analysis of variance it was found that there was no significant difference at the 5% level between any of the extracts from 3-II day old coils. However when data for 2 day old coils was included in the analysis significant differences were found at the I and 5% levels.

When the amount of protein per extract was expressed on a per coil basis the pattern of change with increasing age of the interaction was found to be different (see Fig. 3.I.2b). The mean protein content per coil decreased between 2 and 3 days and 3 and 4 days. There was then a small increase between 4 and 5 days with a moderate fall between 5 and 6 days. There was then a small increase in the mean content per coil between 6 and 7 days with a small decrease between 7 and 8 days, before another rise between 8 and I0 days. This was followed by a large increase between I0 and II days post inoculation. When analysis of variance was used to compare the mean protein contents per coiled region from 2 to I0 days no significant differences were found between them at the 5% level. This

Table 3.1.2 The change in the number of coiled regions per 80mg sample of plant material taken from host/parasite interactions of increasing age.

Age of coiled region (days)	mean number per 80mg sample
2	6.00
3	4.00
4	4.00
5	3.75
6	4.40
7	3.65
8	3.50
10	2.75
11	2.25

Fig. 3.I.2a. The change in the mean protein concentration in extracts of *Cuscuta campestris* coils 2-11 days after inoculation onto *Pelargonium* petioles (I) = standard error bars.

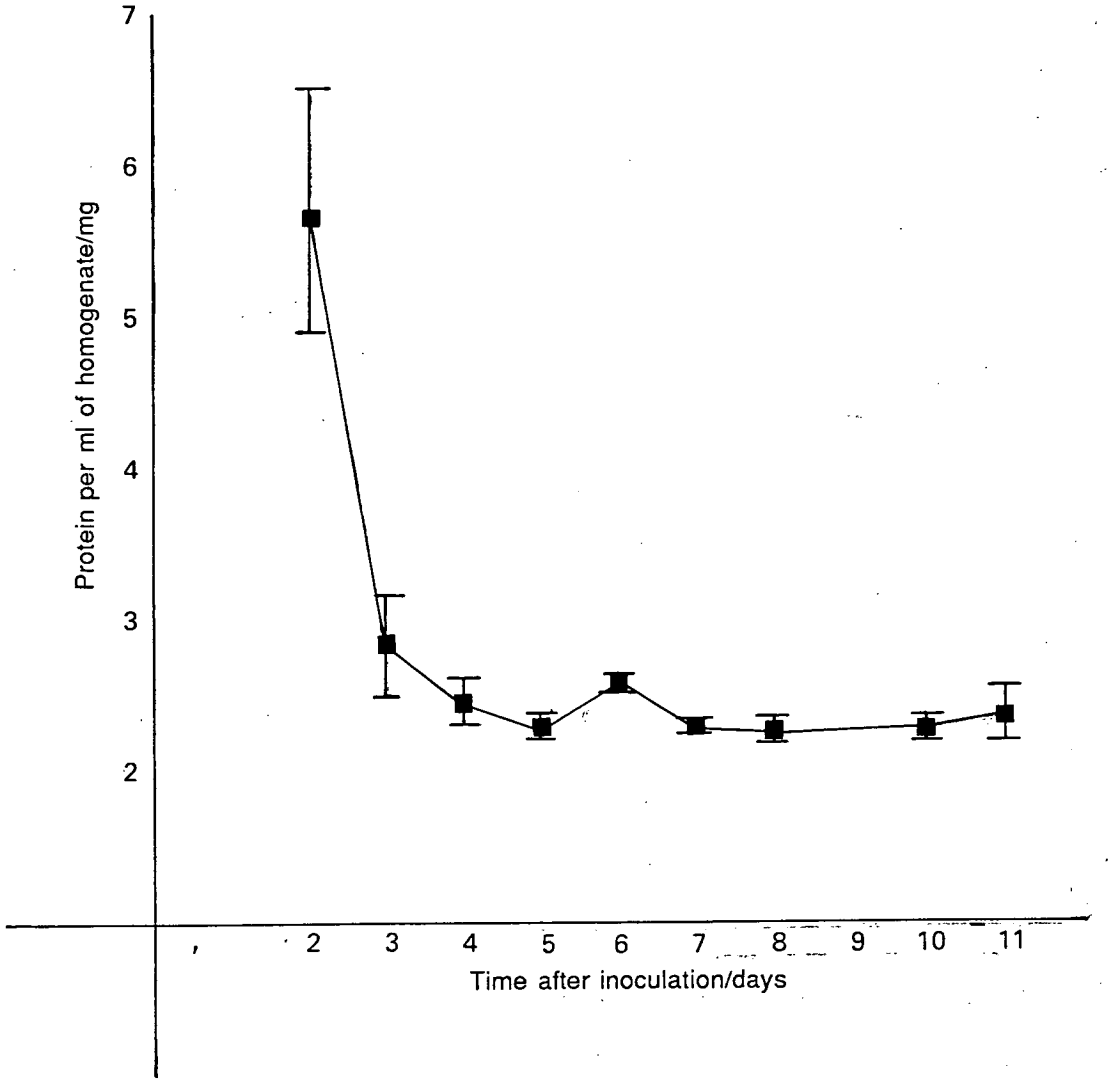
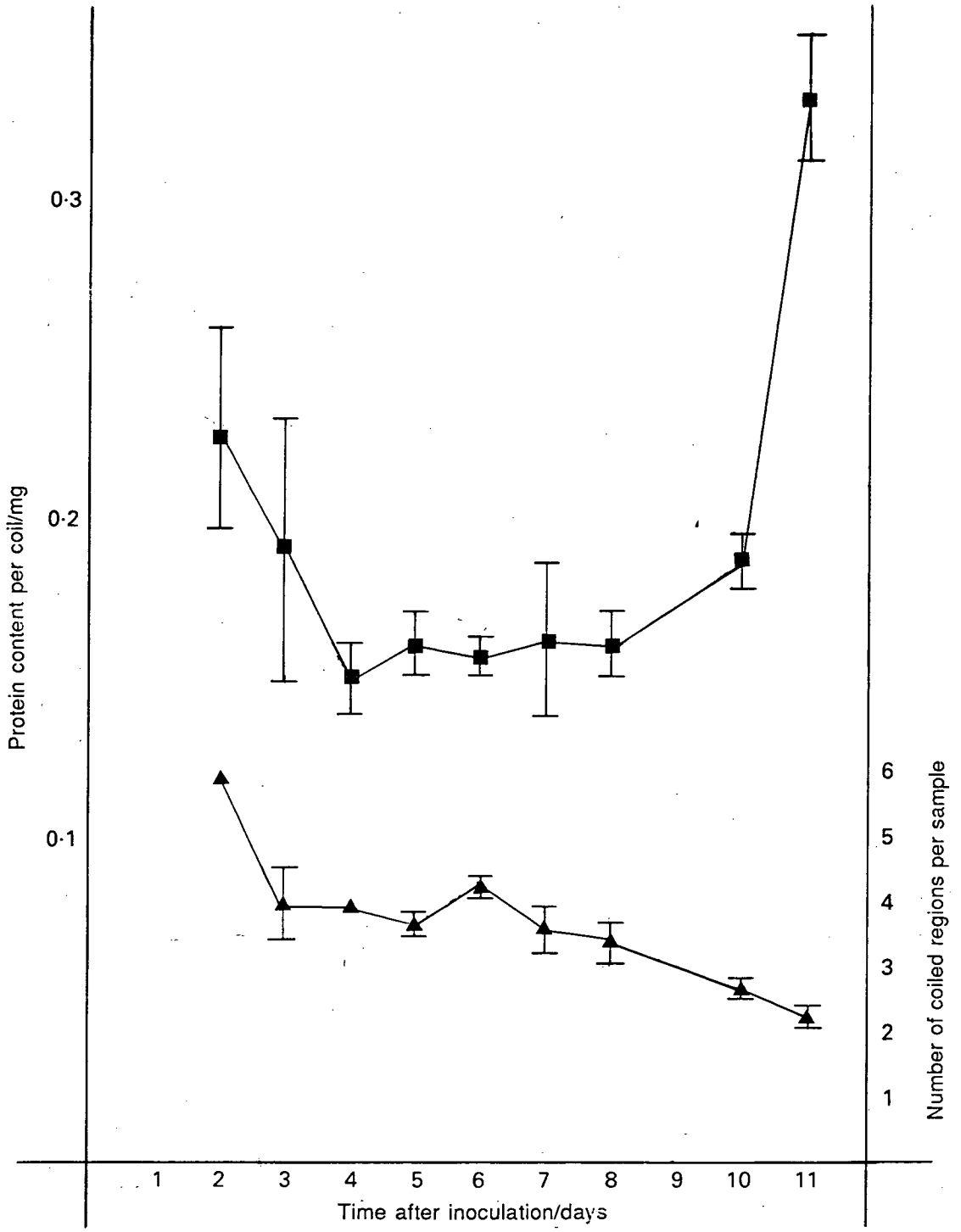


Fig. 3.I.2b. The change in the mean protein content per tight coiled region and the number of coiled regions per 80mg sample 2-II days after inoculation onto *Pelargonium* petioles (■) = protein content (▲) = number of coiled regions (I) = standard error bars.



showed that there was no significant change in the protein content per coil over this period. However, when the values for the protein content per coil after 11 days were included in the analysis significant differences were apparent at the 5 and 1% levels. The protein contents per coiled region could therefore be said to remain relatively constant up to 10 days and then to rapidly increase between 10 and 11 days after inoculation.

The change in cellulase (CE) activity with time

The change in the mean CE activity per ml of extract is shown in Fig 3.1.2c. The activity was low in extracts of 2 day-old coiled regions with rises between 2 and 3 and 3 and 4 days. Over the next 2 days the mean value decreased. The activity then increased again after 7 days before decreases between 7 and 8, 8 and 10 and 10 and 11 days. Analysis of variance showed that the activity changes between 2 and 3, 3 and 4, 5 and 6 and 7 and 8 were significant at the 5% level. The change in activity between 8 and 10 days was significant at the 1% level.

The pattern of change of the mean CE activity per mg protein with time was very similar to that for the activity per ml of homogenate (see Fig. 3.1.2d). Rises and fall of activity occurred on the same days. The standard errors of the mean values were however larger than for the activites per ml on any day. The difference in the pattern of change of CE activity per mg protein and the change in the protein content of extracts with time (see Fig 3.1.2a) showed that the changes in enzyme activity were not directly linked to the quantity of protein present. The changes in mean CE activity between 2 and 3, 3 and 4 and 8 and 10 days were significant at the 5% level. The change in the mean CE activity per coiled region with age of interaction (see Fig 2.1.2e), showed the same two peaked distribution as was shown by the changes in mean activity per ml of crude homogenate and per mg protein. There was however a difference, between 10 and 11 days; there was an increase in the mean cellulase activity per coil. Changes in the activity per coil between 2 and 3 and 3 and 4 days were found to be significant at the 5% level. The changes in activity between days 5 and 6, 7 and 8, 8 and 10 and 10 and 11 were not.

This experiment showed that :-

(1) The fresh weight of the coiled regions increased with time.

(2) The protein concentration decreases initially and then remains constant.

(3) The protein content per coil remained relatively constant between days 2 and 10 and then increased dramatically between days 10 and 11.

(4) During invasion the CE activity in the tight coiled region of the parasite rises to a maximum at about 4 or 5 days and then decreases again.

(5) The changes in CE activity were not directly linked to the changes in the quantity of protein.

(6) The standard errors of the mean values of CE activity per ml extract per g

Fig. 3.I.2c.: The change in the mean cellulase (CE) activity per ml of extract of tightly coiled regions of *Cuscuta campestris* between 2 and II days after inoculation onto *Pelargonium* petioles (I) = standard error bars.

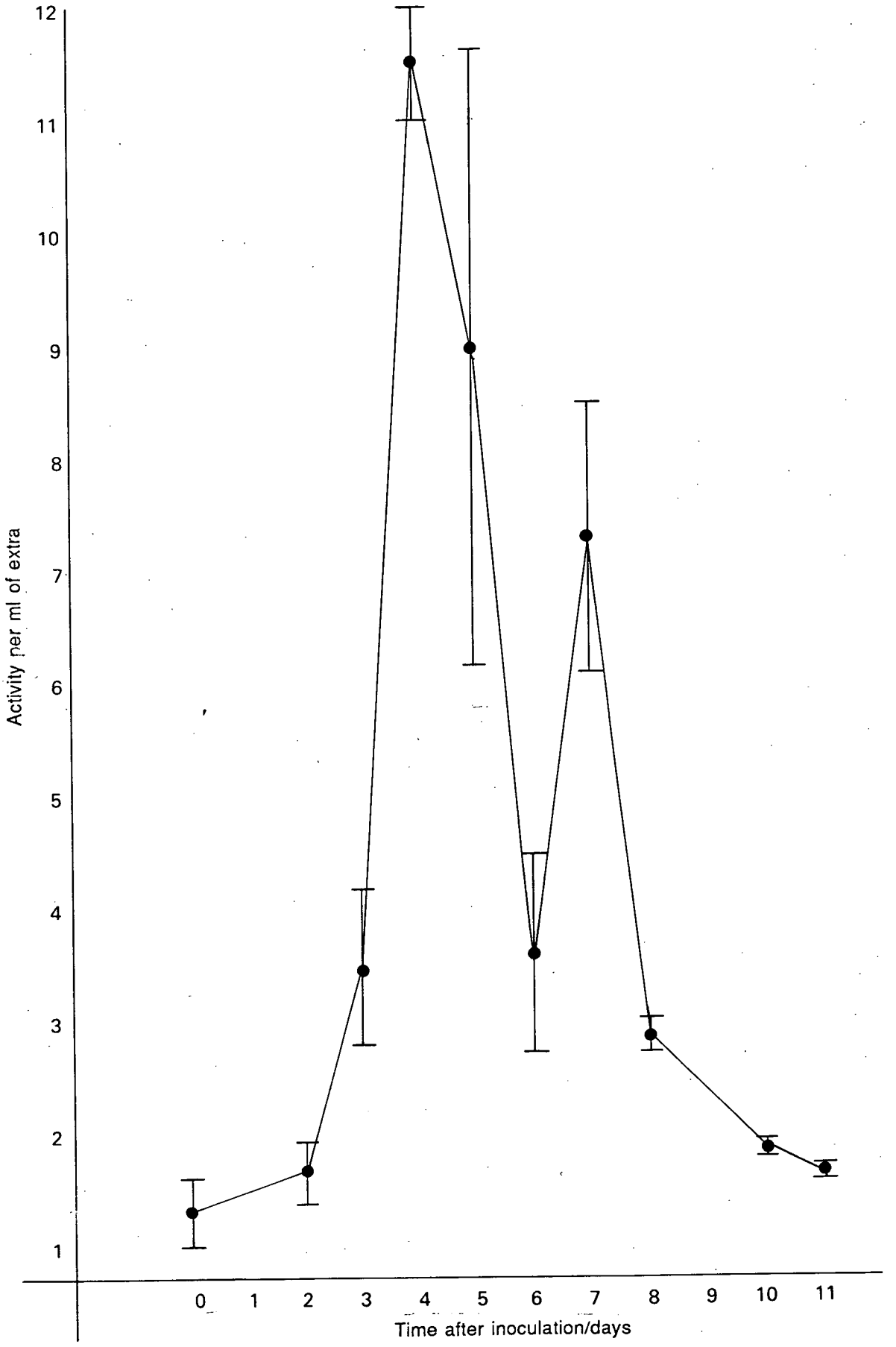


Fig. 3.1.2d. The change in the mean cellulase (CE) activity per mg of protein in extracts of tight coiled regions of *Cuscuta campestris* between 2 and 11 days after inoculation onto *Pelargonium* petioles, (I) = standard error bars.

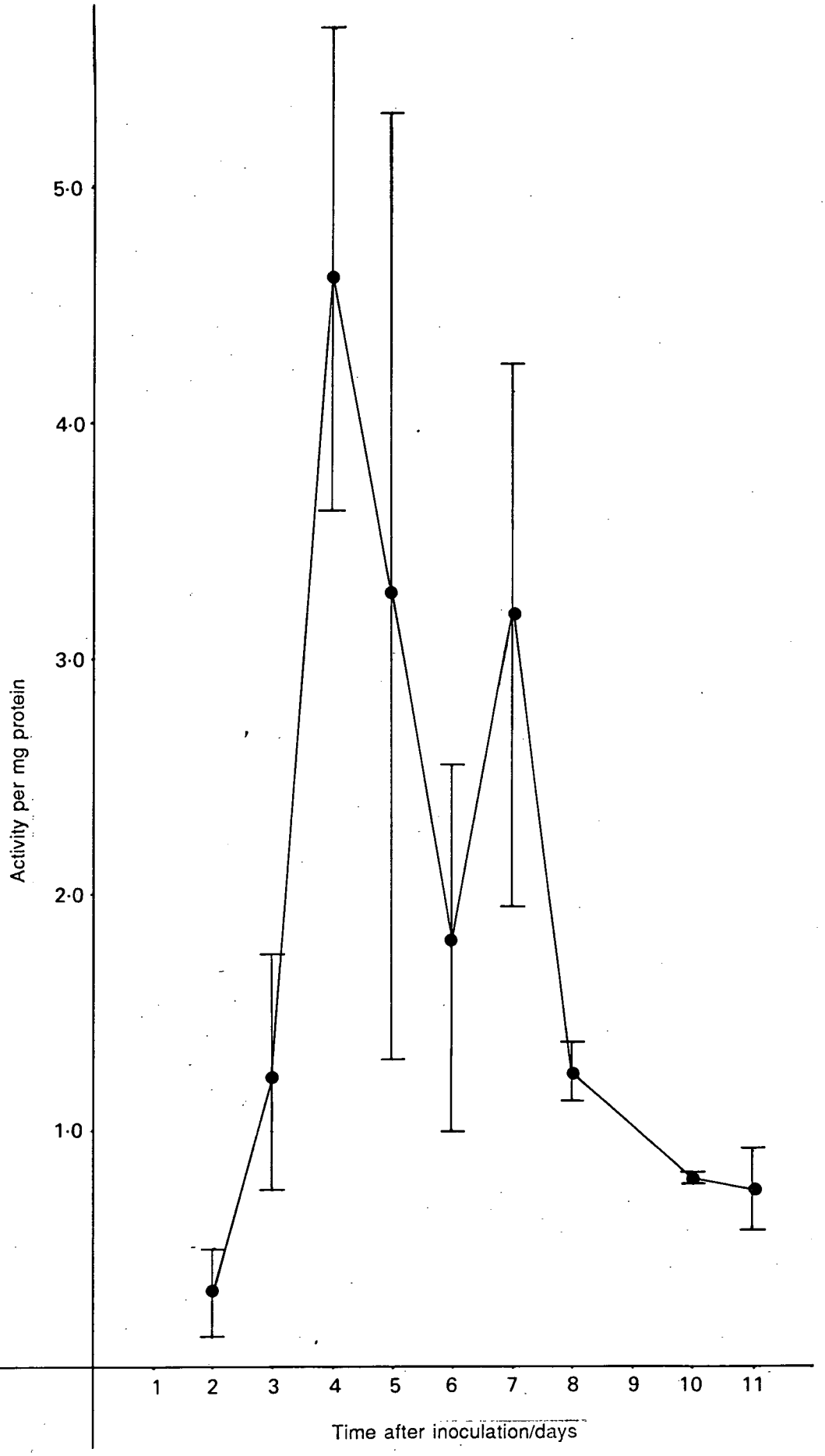
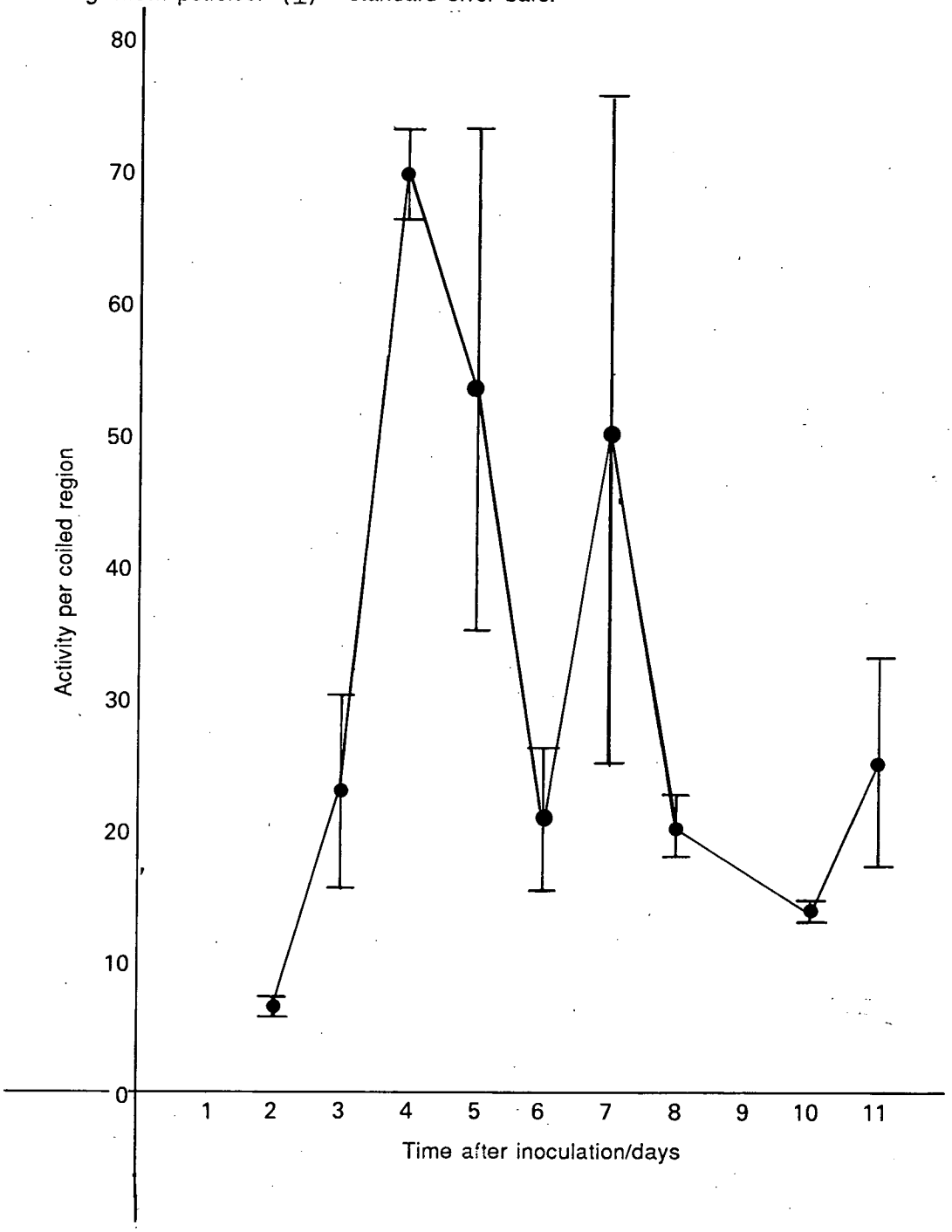


Fig. 3.I.2e. The change in the mean cellulase (CE) activity per coiled region for tight coiled regions of *Cuscuta campestris* between 2 and 11 days after inoculation onto *Pelargonium* petioles. (I) = standard error bars.



protein and per coil were largest on day 5.

Having gathered the above information about the parasite the activity of CE was now measured in the host tissues. Assays were performed on parasitised and unparasitised petioles.

3.I.3 Cellulase (CE) activity in extracts of infected and uninfected *Pelargonium* petioles

The aim of this experiment was two-fold. Firstly to determine the activity of CE in *Pelargonium* petiole tissues. Secondly to see if penetration with *Cuscuta* haustoria caused any change in the activity of the enzyme in the host.

Experimental

Cuscuta coils were carefully dissected off excised *Pelargonium* petioles revealing the bases of the penetrated haustoria. The parts of the petioles containing haustoria were cut out from the rest of the petiole. Penetrated tissue from approximately 20 interactions was divided between 5 samples, each weighing 0.1g. There were approximately 4 interactions represented in each sample.

In addition to the above, five 0.1g samples of green uninfected petioles were excised in the cold room from expanding leaves.

Protein was extracted from each sample by the freeze/thaw method described in section 5.I.2 of Ch2 using Tris/HCl extraction buffer. Viscometric CE assays were performed on 0.Iml aliquots of each protein extract at 23^{o}C. Flow times were recorded after 0, 2.5, 5.0 and 22.5hr. Controls for each sample consisted of aliquots of autoclaved extract mixed with substrate in the same proportions and under the same conditions as the crude extracts and substrate. Amounts of protein in each sample were estimated by assaying 50µl aliquots by Beardens method (see section 5.3.I of Ch2). The CE activities per ml of extract were determined for infected and uninfected petioles. The results are shown in Table 3.I.3c and d. The mean protein concentrations for the petioles are shown in Table 3.I.3d.

The Cellulase activity in the petioles

For uninfected petioles only two of the five samples showed any activity after 22.5 hr (samples 2 and 5 see Table 3.I.3a). Only sample I in the assay of crude extracts of infected petioles showed any activity (sample I, Table 3.I.3b).

The Protein content of the petioles and CE activity per unit protein

The mean protein contents in infected and uninfected petioles were very similar (see Table 3.I.3c). The overlap of the standard errors showed that they were indistinguishable from each other with respect to protein content. The protein concentration was quite low

Table 3.I.3a. The cellulase (CE) activity in crude extracts of petioles of newly expanded *Pelargonium* leaves. In samples I-5 the crude extract was added to the substrate, in samples 6-10 autoclaved extract was added to the substrate (activity in units per ml per hr)

sample	Time (hr)	Activity per ml extract
1	2.50	0.00
	5.00	0.02
	22.50	0.00
2	2.50	0.00
	5.00	0.00
	22.50	0.05
3	2.50	0.00
	5.00	0.01
	22.50	0.00
4	2.50	0.00
	5.00	0.10
	22.50	0.03
5	2.50	0.00
	5.00	0.00
	22.50	0.00
6	2.50	0.03
	5.00	0.03
	22.50	0.00
7	2.50	0.00
	5.00	0.00
	22.50	0.00
8	2.50	0.00
	5.00	0.00
	22.50	0.00
9	2.50	0.00
	5.00	0.00
	22.50	0.04
10	2.50	0.00
	5.00	0.02
	22.50	0.00

Table 3.I.3b. The cellulase (CE) activity in crude extracts of *Pelargonium* petioles containing penetrating haustoria. Samples I-5 contained extract and substrate, samples 6-10 autoclaved extract and substrate. (Activity in units per ml of extract per hr).

sample	Time (hr)	Activity per ml extract
1	2.50	0.00
	5.00	0.02
	22.50	0.03
2	2.50	0.00
	5.00	0.00
	22.50	0.00
3	2.50	0.00
	5.00	0.00
	22.50	0.00
4	2.50	0.00
	5.00	0.00
	22.50	0.00
5	2.50	0.00
	5.00	0.00
	22.50	0.00
6	2.50	0.00
	5.00	0.03
	22.50	0.00
7	2.50	0.00
	5.00	0.01
	22.50	0.06
8	2.50	0.03
	5.00	0.06
	22.50	0.00
9	2.50	0.00
	5.00	0.00
	22.50	0.00
10	2.50	0.00
	5.00	0.00
	22.50	0.00

Table 3.I.3c. The protein concentration in crude extracts of infected and uninfected *Pelargonium* petioles. (se) = standard error

Infected petioles

Sample	Protein ($mg\ ml^{-1}$)	mean	Se.
1	0.358)	0.306	±0.050
2	0.310)		
3	0.167)		
4	0.390)		

Uninfected petioles

Sample	Protein ($mg\ ml^{-1}$)	mean	Se.
1	0.319)	0.317	±0.028
2	0.302)		
3	0.392)		
4	0.256)		

Table 3.1.3d. The cellulase (CE) activty in units per mg protein for extracts of infected and uninfected petioles

petiole type	extract	activity (per mg protein)
infected	1	0.104
uninfected	1	0.072
	2	0.032

as were the CE activities relative to those found in *Cuscuta* stem tissues. The enzyme activity per mg protein was calculated for extracts which had shown some CE activity. These values are shown in Table 3.I.3d The values ranged between 0.032 and 0.I04 units of activity per mg protein per hr.

This experiment showed that little or no CE activity could be measured in extracts of infected or uninfected petioles even after 22.5 hr. The quantity of protein extracted from the two types of petiole under identical conditions was indistinguishable.

The low levels of activity found in this assay and the absence of activity in several samples may have been for at least two reasons:-

(I) The activity of the enzyme in these petioles was indeed very low or

(2) The crude extracts of *Pelargonium* contained an inhibitor which prevented or restricted CE measurement *in vitro.* The latter possibility was examined in the following experiment.

3.1.4 The cellulase (CE) activity in extracts of tight coiled regions of *Cuscuta* coils mixed with extracts of *Pelargonium* petioles

A short series of experiments was designed to discover if crude protein extract of *Pelargonium* petioles contained an inhibitor which affected the measurement of CE *in vitro.*

Protein extracts were prepared from three different types of petiole tissues:-

(I) Uninfected green petioles.

(2) Green petiole tissues containing penetrated *Cuscuta* haustoria.

(3) Senescing petioles containing penetrated haustoria.

The effect of each type of extract on CE activity was examined in separate experiments. Five experiments were performed. Experiment I examined the effect of crude protein extract from green uninfected petioles; 2 and 4 that from green penetrated tissues and 3 and 5 from senescing, penetrated tissues.

Protein was extracted from 0.lg samples of each type of tissue by the freeze/thaw method (see section 5.2.I of Ch.2). The types of reaction mixture (RM) used in each experiment were as follows:

Quantities in ml

COMPONENTS	Reaction mixture (RM) 1	2	3
crude protein extract *(Cuscuta)*	0.05	0.05	-
crude protein extract *(Pelargonium)*	-	0.05	-
Tris/HCl extraction buffer pH 8.1	0.05	-	0.10
1% CMC in phosphate buffer pH 6.1	0.20	0.20	0.20

At least three replicates of each reaction mixture were made up, except for experiment (1) , where there were only two of reaction mixture 3. In each experiment the CE activities in reaction mixtures 1 and 2 were compared. If the *Pelargonium* extract contained some inhibitory factor which affected CE measurement *in vitro* it would be expected that those reaction mixtures which contained it e.g. (RM 2) would have significantly smaller CE activities than those which did not contain it e.g. (RM 1). In each experiment (RM 3) represented the control. This reaction mixture measured the change in the viscosity of the substrate with time during the experiment in the presence of the extraction buffer, but in the absence of the enzyme. If there was any change in the viscosity of the controls during the experiment a 'relative' activity was calculated for this reaction mixture. If the value was large this value was to be subtracted from those obtained for the experimental mixtures to give a true value for the CE activity. The CE activity in each experiment was determined as described in section 6.1 of Ch2. Flow times were recorded at different times in each experiment as follows:

experiment number	recording times in hours
1	0.00, 0.50 + 1.25
2	0.00, 0.67 + 1.25
3	0.00, 0.50, 1.00 + 21.50
4	0.00, 0.58, 1.00 + 2.18
5	0.00, 0.58, 1.18 + 1.83

The CE activity per ml of extract was calculated for each reaction mixture (Table 3.1.4a-e). Means, standard deviations and standard errors were calculated for each group of replicates (Table 3.1.4 f-j). Analysis of variance was used to compare the mean activities of reaction mixtures with or without *Pelargonium* extract.

In experiment 1 samples 9 and 10 showed little or no relative activity. In experiment 2 only one of the controls (sample 8) showed any theoretical activity. The same was true for experiments 4 and 5. In experiment 3 each control showed a small amount of relative activity. Even where the control showed some relative activity the values were an order of magnitude below the experimental samples. Consequently the values were not subtracted from the activities for the experimental reaction mixtures in the presentation of the data. The mean CE activity of RM2 was greater than that for RM1 (Table 3.1.4a). This difference is not however, significant at the 5% level after 0.5 and 1.25hr. The same was true in experiment 2 when the extract used was taken from *Pelargonium* petioles infected with parasite. Experiment 4 was a repeat of experiment 2. The mean activity of the RM2 was slightly less in two readings and slightly larger in the third than that for RM1 (Table 3.1.4d). None of the differences were significant at the 5% level. None of the differences between RM1 and 2 in experiment 2 or 4 were found to be significant at the 5% level either.

In experiment 3 the mean CE activity of RM2 (Table 3.1.4h) was larger than that of RM1 at all the times of measurement (Table 3.1.4h). Experiment 5 was a repeat of experiment 3. After 0.88hrs the mean activity of RM2 which contained *Pelargonium* extract was lower than that of RM1 which did not contain it. These differences in the mean activity are significant at the 5% level. Further measurements were taken after 1.18 and 1.83 hr. These differences in the mean activities of RM1 and 2 are not significant at the 5% level.

This experiment showed that the addition of crude protein extract of penetrated or unpenetrated *Pelargonium* petioles did not significantly affect the measurement of CE *in vitro*. The same was true for extracts of senescent penetrated petioles in the majority of cases.

Table 3.I.4a The cellulase (CE) activity in different reaction mixtures from experiment (1) in section 3.I.4 C = crude protein extract of *Cuscuta* coils P = crude protein extract of green uninfected *Pelargonium*, E = extraction buffer and S = substrate.

sample	reaction mixture	Time (hr)	Activity per ml of extract
1	C+E+S	0.50	6.36
		1.25	4.00
2	C+E+S	0.50	5.24
		1.25	4.26
3	C+E+S	0.50	5.32
		1.25	4.94
4	C+E+S	0.50	5.24
		1.25	3.62
5	C+P+S	0.50	6.72
		1.25	6.18
6	C+P+S	0.50	5.08
		1.25	3.60
7	C+P+S	0.50	7.02
		1.25	5.14
8	C+P+S	0.50	8.20
		1.25	7.07
9	E+S	0.50	0.00
		1.25	0.14
10	E+S	0.50	0.38
		1.25	0.38

Table 3.I.4b The cellulase activity (CE) in different reaction mixtures from experiment (2) in Section 3.I.4 C = crude extract of *Cuscuta* coils, P = crude protein extract of green *Pelargonium* petioles containing haustoria, E = extraction buffer and S = substrate.

sample	reaction mixture	Time (hr)	Activity per ml of extract
1	C+E+S	0.67	3.85
		1.25	3.57
2	C+E+S	0.67	4.15
		1.25	3.85
3	C+E+S	0.67	3.67
		1.25	3.59
4	C+E+S	0.67	3.76
		1.25	2.85
5	C+P+S	0.67	7.40
		1.25	6.11
6	C+P+S	0.67	4.98
		1.25	7.43
7	C+P+S	0.67	2.85
		1.25	2.82
8	E+S	0.67	0.00
		1.25	0.00
9	E+S	0.67	0.38
		1.25	0.38
10	E+S	0.67	0.00
		1.25	0.00

Table 3.I.4c The cellulase (CE) activity in different reaction mixtures from experiment (3) in section 3.I.4. C = crude protein extract of *Cuscuta* coils P = protein extract of senescent *Pelargonium* petioles containing penetrated haustoria, E = extraction buffer, S = substrate.

sample	reaction mixtures	Time (hr)	Activity per ml of extract
1	C+E+S	0.50	4.44
		I.00	5.62
		2I.50	1.26
2	C+E+S	0.50	4.44
		1.00	4.32
		2I.50	6.42
3	C+E+S	0.50	4.24
		I.00	5.40
		2I.50	0.95
4	C+P+S	0.50	5.00
		I.00	5.80
		2I.50	0.98
5	C+P+S	0.50	7.83
		I.00	6.42
		2I.50	0.95
6	C+P+S	0.50	4.20
		I.00	4.02
		2I.50	0.80
7	E+S	0.50	0.10
		I.00	0.23
		2I.50	0.0I
8	E+S	0.50	0.28
		1.00	0.05
		21.50	0.00
9	E+S	0.50	0.00
		1.00	0.20
		21.50	0.01

Table 3.1.4d The cellulase (CE) activity in different reaction mixtures from experiment (4) of section 3.I.4 C = crude protein extract of *Cuscuta* coils P = crude protein extract of green *Pelargonium* petioles containing penetrating haustoria, E = extraction buffer, S = substrate

sample	reaction mixture	Time (hr)	Activity per ml of extract
1	C+E+S	1.00	7.10
		1.58	5.41
		2.18	5.69
2	C+E+S	1.00	4.65
		1.58	4.50
		2.18	4.21
3	C+E+S	1.00	3.67
		1.58	3.78
		2.18	3.58
4	C+E+S	1.00	7.60
		1.58	6.03
		2.18	4.96
5	C+E+S	1.00	3.24
		1.58	3.22
		2.18	3.05
6	C+P+S	1.00	6.02
		1.58	5.22
		2.18	5.15
7	C+P+S	1.00	5.18
		1.58	4.88
		2.18	6.28
8	C+P+S	1.00	4.76
		1.58	4.16
		2.18	3.76

sample	reaction mixture	Time (hr)	Activity per ml of extract
9	C+P+S	1.00	4.12
		1.58	3.43
		2.18	3.04
10	C+P+S	1.00	5.12
		1.58	4.98
		2.18	4.90
11	E+S	1.00	0.12
		1.58	0.00
		2.18	0.03
12	E+S	1.00	0.00
		1.58	0.00
		2.18	0.00
13	E+S	1.00	0.00
		1.58	0.00
		2.18	0.00

Table 3.I.4e The cellulase (CE) activity in different reaction mixtures from experiment (5) of section 3.I.4 C = crude protein extract of *Cuscuta* coils, P = crude protein extract of senescent *Pelargonium* petioles containing penetrating haustoria, E = extraction buffer, S = substrate.

sample	reaction mixtures	Time (hr)	Activity per ml of extract
1	C+E+S	0.58	5.20
		1.18	3.77
		1.83	3.74
2	C+E+S	0.58	4.44
		1.18	4.62
		1.83	4.05
3	C+E+S	0.58	5.24
		1.18	4.62
		1.83	3.82
4	C+E+S	0.58	6.37
		1.18	5.35
		1.83	4.45
5	C+E+S	0.58	5.00
		1.18	3.77
		1.83	2.92
6	C+P+S	0.58	3.64
		1.18	3.22
		1.83	3.03
7	C+P+S	0.58	3.62
		1.18	3.23
		1.83	2.69
8	C+P+S	0.58	4.12
		1.18	4.06
		1.83	3.35
9	C+P+S	0.58	4.65
		1.18	4.06
		1.83	3.72

Contd.

sample	reaction mixtures	Time (hr)	Activity per ml of extract
10	C+P+S	0.58	4.37
		1.18	4.42
		1.83	3.58
11	E+S	0.58	0.00
		1.18	0.00
		1.83	0.00
12	E+S	0.58	0.00
		1.58	0.00
		1.18	0.22
		1.83	0.20
13	E+S	0.58	0.00
		1.18	0.00
		1.83	0.00

Table 3.1.4f The means, standard deviations (s.d.) and standard errors (s.e.) of groups of replicates from experiment (5). C=crude extract from *Cuscuta* coils P=crude extract from senescent petioles of *Pelargonium* containing penetrated haustoria, E=extraction buffer, S=substrate.

reaction mixtures	Time (hr)	mean Activity	s.d.	s.e.
C+E+S	0.58	5.25	0.70	0.31
	1.18	4.43	0.67	0.30
	1.83	3.80	0.56	0.25
C+P+S	0.58	4.08	0.45	0.20
	1.18	3.80	0.54	0.24
	1.83	3.27		

Table 3.1.4g The means, standard deviations (s.d.) and standard errors (s.e.) of groups of replicates from experiment (1) C=Crude protein extracts of *Cuscuta* coils, E=extraction buffer, P=crude protein extracts from green *Pelargonium* petioles, S=substrate.

reaction mixtures	Time (hr)	mean Activity	s.d.	s.e.
C+E+S	0.50	5.54	0.55	0.27
	1.25	4.20	0.56	0.28
C+P+S	0.50	6.76	1.29	0.64
	1.25	5.50	1.49	0.75

Table 3.1.4h The means, standard deviations (s.d.) and standard errors (s.e.) of groups of replicates from experiment (2). C=crude extract from *Cuscuta* coils, P=crude extract from petioles of *Pelargonium* penetrated the haustoria, E=extraction buffer, S=substrate.

reaction mixtures	Time (hr)	mean Activity	s.d.	s.e.
C+E+S	0.67	3.86	0.21	0.10
	1.25	3.47	0.43	0.21
C+P+S	0.67	5.23	2.05	1.19
	1.25	5.45	2.37	1.37

Table 3.1.4i The means, standard deviations (s.d.) and standard errors (s.e.) of groups of replicates from experiment (3). C=crude extracts from *Cuscuta* coils, P=crude extracts from green petioles of *Pelargonium* containing penetrated haustoria, E=extraction buffer, S=substrate.

reaction mixtures	Time (hr)	mean Activity	s.d.	s.e.
C+E+S	0.50	4.37	0.12	0.07
	1.00	5.11	0.70	
	21.50			
C+P+S	0.50	5.68	1.91	1.10
	1.00	5.41	1.25	0.72
	21.50			

Table 3.1.4jj The means, standard deviations (s.d.) and standard errors (s.e.) of groups of replicates from experiment (4). C=crude extract from *Cuscuta* coils P=crude extract from senescent petioles of *Pelargonium* containing penetrated haustoria, E=extraction buffer, S=substrate.

reaction mixtures	Time (hr)	mean Activity	s.d.	s.e.
C+E+S	1.00	5.25	1.99	0.89
	1.58	4.59	1.15	0.51
	2.18	4.10	0.79	0.35
C+P+S	1.00	5.04	0.69	0.31
	1.58	4.53	0.73	0.33
	2.18	4.63		

Little or no CE activity was detectable in green *Pelargonium* petioles with or without invading haustoria using the viscometric method. The previous experiment showed that this was not due to an inhibitor. One other possibility for not measuring any CE in the petioles was that viscometric method may not have been suitable for detecting this enzyme in *Pelargonium* tissues. Alternatively CE may have been present in the petioles but at too low concentrations to be detected in the assay. Petioles may not be expected to be particularly metabolically active. Other parts of the *Pelargonium* plant such as the activity growing shoot apex, would be expected to have a higher CE activity. If the assay procedure of section 3.I.3 was repeated using these metabolically more active tissues and CE activity was found, it would suggest that the method was not at fault. On the contrary it would suggest that the enzyme activity was simply low in the petiole tissues. The assay procedure of section 3.I.3 was therefore repeated using extracts of *Pelargonium* shoot apices. The experiment is described below.

3.I.5 Cellulase (CE) activity in extracts of growing shoot apices from *Pelargonium* plants

The aim of this experiment was to show that CE could be detected in *Pelargonium* tissues using the viscometric method.

In this experiment the assay procedure was the same as that used in section 3.I.3 except that the extract was made from *Pelargonium* shoot apices in which cell wall degrading enzymes would be expected to be present in large quantities.

Apices plus the last two unexpanded leaves of *Pelargonium* stem apices were excised in the cold room. Protein was extracted from four, 0.lg samples of these tissues by the freeze/thaw method (see section 5.I.2 of Ch2). CE assays (see section 6.I Ch.2) were performed on 0.lml aliquots of each sample of extract. Controls consisted of extraction buffer mixed with substrate in the same proportions and under the same conditions as apical extracts mixed with substrate. Flow times of the reaction mixtures were recorded after 0, I, I.5, 3.5 and 5.5 hr. Cellulase activity was expressed per ml of extract (see Table 3.I.5a). Mean values, standard deviations and standard errors were also calculated.

Results

The viscosity of the controls changed with time during the assay procedure. This was taken into consideration in working out the activities in the experimental extracts as seen in Table 3.I.5a. CE activity was found in all four extracts of the apices (Table 3.I.5a). The range of activity per ml of extract was quite large (after 2.5. hr of incubation it ranged between 0.I6 and 2.I3 units per ml of extract). As protein estimations were not performed it was not possible to determine if this was related to the amount of protein present.

Table 3.1.5a The cellulase (CE) activity per ml of extract of *Pelargonium* shoot apices measured with a viscometric method. (Units of activity per ml of extract per hr).

sample	Time of incubation				
	30 mins	1 hr	2 hr 15 min	3 hr 30 min	5 hr 30 min
1	0.00	0.39	0.30	0.43	0.27
2	0.00	0.39	0.37	0.31	0.20
3	0.00	0.00	0.08	0.00	0.05
4	0.00	0.16	0.19	0.09	0.04
5	0.00	0.00	0.38	0.29	0.11
mean	0.00	0.19	0.26	0.24	0.13

Summary from section 3.I.I-5

The following points emerged from the last three experiments:-

(I) Little or no CE activity was measurable in extracts of *Pelargonium* petioles using the viscometric assay procedure.

(2) Crude extracts of *Pelargonium* do not contain an inhibitor which affects CE measurement *in vitro.*

(3) When the assay procedure used in section 3.I.2 was repeated using actively growing apical tissues, much higher CE activities were recorded.

These experiments would suggest that the reason for not detecting CE activity in *Pelargonium* petioles was the very low endogenous activity.

The petiole is the part of the *Pelargonium* plant which has been chosen to be the host *in vitro.* Each one is supported at either end in a medium which contains high concentrations of growth substance (see section 2). Application of auxins has been shown in other systems to stimulate CE activity. It is possible that the culture procedure could change the activity of CE in the cells of the petiole and affect the ability of *Cuscuta* shoot tips to infect.

This section is concerned with the changes in cell-wall degrading enzymes during or as a result of parasite invasion. It is important to determine whether the culture procedure itself caused changes in the enzyme activity before the *in vitro* system could be used to investigate any changes which were the result of parasitism.

An experiment was performed to see if the activity of CE was elevated as a result of the culture procedure. The experiment is described below:

3.I.6 The cellulase (CE) activity in extracts of *Pelargonium* petioles which had been cultured for 0, 3 and 5 days.

The aim of this experiment was to see if cellulase (CE) activity was stimulated when *Pelargonium* petioles were cultured for 3 and 5 days.

Petioles from newly emerged leaves were excised, sterilised (see section 3 of Ch2), trimmed and cultured (see section 3.7 of Ch2) with two to each container. The containers were sealed with parafilm and incubated under the usual conditions (see section 2 of Ch3). Ten petioles were harvested after three days in culture and a further ten after 5 days. The same number of 60mm petioles were harvested from newly emerged leaves of greenhouse plants.

The 10mm end portions of the cultured petioles (the regions of most expansion during culture (see section 2.3.3. of Ch3)) were removed from the rest of the petiole. The two regions of the petioles were extracted and assayed separately. Protein was extracted using the freeze/thaw method with Tris/HCl extraction buffer (see section 5.I.2. of Ch2).

Extracts were made from four 0.2g samples of end and middle portions of petioles cultured for 3 and 5 days. Five samples of fresh petioles were extracted. CE assays were performed on 0.lml aliquots of each extract at 23°C. Flow times were recorded after 0, 2.5, 5.0 and 22.5 or 24.0 hr (3 days cultured petioles 24hr and 5 day cultured and fresh petioles 22.5hr). Controls consisted of substrate mixed with boiled extract. The amount of protein in each extract was estimated by Bearden's method (see section 5.3.l Ch2).

Results - The changes in the flow times

The flow times for the reaction mixtures are shown in Table 3.1.6a. Those which contained extract of the end portions of petioles cultured for three days increased with time during the assay. Their controls showed slight decreases in their flow times after 24hr. One of the replicates had a progressively smaller flow time as the assay proceeded. Its control showed a slight increase in flow time after 24hr. The decrease in the viscosity of the replicate corresponded to an activity of 0.l4, 0.l3 and 0.054 units per ml of extract after 2.5, 5 and 24hr respectively. The flow times of three of the reaction mixtures containing the middle portions of petioles for 3 days increased or stayed the same with time. The flow times of replicate 4 decreased with time and its control increased slightly. This fall in flow time corresponded to activities of 0.48, 0.30 and 0.07l units per ml of extract after 2.5, 5 and 24hr respectively. The flow times of all the reaction mixtures containing extract of the end pieces of petioles cultured for five days decreased with time during the assay. The controls of replicates l, 2 and 3 did not change with time. The control of replicate 4 did decrease with time but not as much as that of the experimental mixture. The activities to which these flow times correspond are shown in Table 3.l.6b. Only replicate 6 of the end piece extracts had measurable CE activity throughout the whole of the experiment. Replicates 2, 3 and 4 had activity by the end of the assay period. Extracts of middle portions of 5 day cultured petioles also had some CE activity, replicate l showed CE activity at all the times that measurements were taken. Replicate 2 showed some activity after 22.5hr. Replicates 3 and 4 had no measurable activity. Some of the extracts of the freshly excised petioles had some small CE activity. Replicates 2 and 5 had measurable activity after 22.5hr. The activity found at 5hr was not evident at 22.5hr and may have been an anomoly. From the available data mean values were obtained for the CE activities in the petioles receiving different treatments after 22.5 or 24 hr. The results are shown in Table 3.l.6c. This table shows the proportion of replicates receiving each treatment which showed any measurable activity. Extracts of end pieces of five day cultured petioles had the largest frequency of the presence of measurable activity. The extracts of freshly excised petioles came next and those of 3 day cultured petioles a close third.

Table 3.1.6a The flow times of the reaction mixtures during the assay measuring CE activity in freshly excised and 3 and 5 day cultured petioles. Replicates recorded with a 'b' are controls. Flow times are measured in seconds.

days in Culture	Region of Petioles	Replicates	Time (hr) 0	2.5	5.0	22.5	24.0
0	whole	1	34.5	35.2	34.0	34.2	-
0	"	2	39.0	41.5	41.2	36.7	-
0	"	3	35.0	36.0	37.0	35.2	-
0	"	4	36.5	37.2	34.0	33	-
0	"	5	35.0	37.2	36.2	34.2	-
0	"	1b	44.0	46.5	43.0	39.5	-
0	"	2b	43.5	43.5	46.2	43.5	-
0	"	3b	46.5	46.0	47.0	39.2	-
0	"	4b	44.0	44.5	45.0	37.2	-
0	"	5b	39.0	39.0	38.5	39	-
0	Ends	1	43	40.5	38.5	-	34.7
3	"	2	50	51.0	51.0	-	51.0
3	"	3	34	33.5	35.2	-	36.2
3	"	4	44.5	46.0	48.0	-	50.7
3	"	1b	39.0	39.5	38.7	-	34.7
3	"	2b	43.0	41.7	42.0	-	51.0
3	"	3b	-	-	-	-	36.2
3	"	4b	49.0	39.5	47.5	-	50.7
3	Middle	1	39.0	38.5	40.0	-	43.0
3	"	2	37.0	36.2	37.7	-	36.7
3	"	3	32.0	33.5	33.7	-	36.0
3	"	4	38.0	31.2	31.2	-	30.5
3	"	1b	55.0	57.5	57.5	-	54.7
3	"	2b	42.0	43.0	42.0	-	40.5
3	"	3b	45.0	45.5	46.0	-	46.0
3	"	4b	46.0	45.7	50.5	-	48.5

Contd.

days in Culture	Region of Petioles	Replicates	Time (hr) 0	2.5	5	22.5	24
5	middle	1	36.5	35.7	34.7	34.0	-
5	"	2	39.5	41.5	40.5	36.1	-
5	"	3	38.0	37.0	40.0	39.0	-
5	"	4	47.0	44.0	48.7	46.2	-
5	"	1b	50.0	51.7	55.5	50.5	-
5	"	2b	47.5	48.0	47.5	44.5	-
5	"	3b	40.5	39.7	40.0	35.0	-
5	"	4b	47.5	45.7	46.0	43.0	-
5	Ends	1	33.5	32.2	33.0	28.0	-
5	"	2	39.5	39.0	39.7	37.0	-
5	"	3	40.0	40.0	39.7	35.0	-
5	"	4	46.5	40.0	35.0	28.5	-
5	"	1b	50.5	50.5	54.7	51.0	-
5	"	2b	63.0	63.5	62.7	63.5	-
5	"	3b	38.0	-	38.5	36.5	-
5	"	4b	46.5	45.0	43.0	38.5	-

Table.3.1.6b The cellulase (CE) activity in extracts of freshly excised and 3 and 5 day cultured *Pelargonium* petioles. E = end region M = middle region W = whole petiole. (units per ml extract).

Days in culture	Region of petiole	Replicate	Activity (units per ml of extract 2.5hr	5 .0hr	22.5hr
5	E	1	0.116	0.022	0.069
5	E	2	0.032	-	0.041
5	E	3	-	-	0.031
5	E	4	-	0.296	0.096
5	M	1	0.064	0.072	0.023
5	M	2	-	-	0.026
5	M	3	0.016	-	-
5	M	4	-	-	-
0	W	1	-	-	-
0	W	2	-	-	0.018
0	W	3	-	-	-
0	W	4	-	0.104	-
0	W	5	-	-	0.008
3	E	1	0.136	0.132	0.057
3	E	2	-	-	-
3	E	3	-	-	-
3	E	4	-	-	-
3	M	1	0.032	-	-
3	M	2	0.060	-	-
3	M	3	-	-	-
3	M	4	0.568	0.302	0.070

Table 3.1.6c. The frequency of the presence of measurable cellulase activity and the mean values of activity for extracts of cultured *Pelargonium* petioles.(w) =whole petiole, (E) = end portion of petiole and (M) = middle portion of petiole

Days in culture	region	frequency of finding measurable activity	mean activity
0	W	2/5	0.013
3	M	1/4	(0.070)
3	E	1/4	(0.057)
5	M	2/4	0.048
5	E	4/4	0.047

The protein content of the petiole extracts is shown in Table 3.I.6d. The end regions of cultured petioles had lower mean protein concentrations than the middle portions. It was not known whether this was a general phenomenon for *Pelargonium* petioles or as the result of the culture procedure because fresh petioles were extracted as a whole and not separated out into end and middle portions. The mean protein content of fresh petioles was greater than the combined mean protein content for petioles cultured for three days, but smaller than that for petioles cultured for five days.

Table 3.I.6e shows the activities of the enzyme extracts per mg protein. In replicates where activity was detected throughout the experiment the activity was found to decrease with time. There was an exception in replicate 2 of the extracts of end pieces of five-day cultured petioles. The relationship between the activities in extracts from petioles with different treatments was the same as described for the activity per ml of extract.

This experiment showed that:-

(I) Some extracts of *Pelargonium* petioles whether fresh or cultured for three or five days contained some measurable CE activity.

(2) From the available data the mean CE activity was greater in extracts of cultured than freshly excised petioles (when activity was found at all).

(3) The frequency of finding measurable activity was greatest in the end portions of five-day cultured petioles and least in three- day cultured petioles.

(4) The enzyme activities were low ranging between 0.036 and I.07 units of activity per mg protein per hour. The majority of the activities were between 0.I and 0.4 units per mg protein per hour.

(5) The protein concentration in the middle portions of the cultured petioles were found to be greater than those in the end portions.

Having investigated CE activity, attention was next turned to the pectin degrading enzymes. The first to be investigated was Pectin Esterase (PE). This enzyme has often been found in plants parasitised by fungi in combination with polygalacturonase (PGURASE).

3.2 Pectin esterase activity in host and parasite

3.2.I Pectin esterase (PE) activity in extracts of tight coiled stems of *Cuscuta campestris*

The aim of this experiment was to establish whether PE was present in coiled *Cuscuta campestris* stem tissues.

Complete coiled regions, bearing penetrating haustoria were dissected from excised *Pelargonium* petioles in the cold-room. Protein was extracted with Tris/HCl extraction buffer by the freeze/thaw method from five, 0.2g samples of coiled regions of stem. The

Table 3.1.6d The protein concentrations in extracts of different parts of freshly excised and 3 and 5 day cultured petioles. W = whole petiole E = end portions (10mm) M = middle portion

Days in Culture	Part of petiole	Replicate	Protein (mg ml^{-1}) concentration
0	W	1	0.14
0	W	2	0.38
0	W	3	0.32
0	W	4	0.39
0	W	5	0.32
3	E	1	0.07
3	E	2	0.14
3	E	3	0.21
3	E	4	0.14
3	M	1	0.16
3	M	2	0.20
3	M	3	0.24
3	M	4	0.28
5	E	1	0.15
5	E	2	0.23
5	E	3	0.26
5	E	4	0.09
5	M	1	0.23
5	M	2	0.26
5	M	3	0.50
5	M	4	0.22
0	W	6	0.12
0	W	7	0.14
0	W	8	0.23
0	W	9	0.15
0	W	10	0.40
0	W	11	0.18

Table 3.I.6e. The CE activity in different portions of freshly excised and 3 and 5 day cultured *Pelargonium* petioles per mg of extracted protein. E = end portions M = middle portions W = whole petiole.

Days in Culture	Part of petiole	Replicate	Activity (units per mg protein) 2.5 hr	5.0 hr	22.5 hr	24.0 hr
0	W	1	-	-	-	-
O	W	2	-	-	0.080	-
0	W	3	-	-	-	-
0	W	4	-	0.470	-	-
0	W	5	-	-	0.036	-
3	E	1	-	-	-	-
3	E	2	-	-	-	-
3	E	3	-	-	-	-
3	E	4	1.000	0.930	-	0.390
3	M	1	-	-	-	-
3	M	2	-	-	-	-
3	M	3	-	-	-	-
3	M	4	1.710	1.070	-	0.250
5	E	1	0.770	0.150	0.460	-
5	E	2	-	-	0.180	-
5	E	3	-	-	0.120	-
5	E	4	-	3.290	1.070	-
5	M	1	0.280	0.310	0.100	-
5	M	2	-	-	0.100	-
5	M	3	0.032	-	-	-
5	M	4	-	-	-	-

crude protein extracts from each sample were pooled together before being purified by the spun column method (see section 5.2.I of Ch2). The presence of PE in the extract was established using the method described in section 6.3(b) of Ch.2. Three replicates of each reaction mixture were made up. Flow times were measured after 0, 0.5, I.5 and I4 hr. (see Table 3.2.Ia).

PE activity

The viscosity of the reaction mixtures which contained protein and substrate (PBS) only showed modest changes with time (see Table 3.2.Ia). The same was true for mixtures of acetate buffer and substrate (BS) and buffer, calcium buffer and substrate (CABS). However, when calcium buffer was added to a reaction mixture containing protein and substrate (PCABS), the viscosity increased rapidly with time. The first replicate of this type of reaction mixture became solid before a flow time measurement for time zero could be made (solidification made it impossible to determine the viscosity of the mixture by measuring the time taken to fall between two points inside a pipette). The other two replicates (PCABS 2 and 3) gave high initial flow times and then became completely solid within 30 minutes.

The initial flow times of replicates of each type of reaction mixture were similar. However, different types of reaction mixture had different initial flow times. The reaction mixtures containing calcium ions all had higher initial flow times than those which did not (see Table 3.2.Ia).

This experiment showed that the presence of calcium ions moderately increased the viscosity of PGURA. This presumably occurred by cross linking the available carboxyl groups. An increase in viscosity with time and complete solidification only occurred when protein extracts and calcium ions were mixed together with the substrate. Solidification of solutions of pectin associated with a combination of Ca^{2+} ions and protein is a standard test for detecting PE activity. Consequently it was concluded that PE was present in coiled regions of *Cuscuta* stem.

Having found PE in coiled tissues, assays were performed on other types of stem tissues.

3.2.2 Pectin esterase activity in extracts of non-coiled *Cuscuta* shoots

The aim of this experiment was to see if PE activity was present in extracts of non-coiled *Cuscuta* stem tissues.

Pieces of *Cuscuta* stem at least 30mm from the apex or from any node were excised from vines grown in the greenhouse. Protein was extracted from three, 0.lg samples by the freeze/thaw method using Tris/HCl extraction buffer. The presence of PE in the crude extracts was determined by the method described in (section 6.3a of Ch2.). Flow times

Table 3.2.1a The flow times of reaction mixtures from section 3.2.1. BS = acetate buffer and substrate PBS = Protein in acetate buffer and substrate, CABS = calcium buffer and substrate and PCABS = Protein plus calcium buffer and substrate.

Reaction mixture	Replicate	Flow time (sec)			
		0 min	35 min	90 min	24 hr
PCABS	1	solid	-	-	-
	2	57.0	solid	-	-
	3	44.0	solid	-	-
CABS	1	33.5	34.5	33.5	29.0
	2	32.0	33.5	32.0	26.5
	3	32.0	34.5	32.5	29.0
PBS	1	21.0	20.0	18.0	16.0
	2	19.5	19.5	18.5	15.5
	3	21.0	20.0	21.0	18.0
BS	1	20.0	20.0	19.0	22.0
	2	21.5	22.0	20.0	22.0
	3	22.0	22.5	22.0	22.0

were measured after 0, 6, 7 and 24 hours. Protein estimations were performed on three (10µl) aliquots of each extract by the Bearden method (see section 5.3.1).

PE Activity

All the crude extracts contained between 1.12 and 2.51 mg protein per ml (Table 3.2.2b). The reaction mixtures containing crude extract (CX) and PGURA became solid and opaque within 7 hrs.

The viscosity of reaction mixtures which contained crude extract which had been boiled before being mixed with the substrate (CXB) did not change even after 24hrs (see Table 3.2.2a). Boiling the crude extract had removed its ability to make the substrate solidify. Such conditions denature proteins. It was concluded that it was the protein component of the crude extract which was responsible for the solidification of the reaction mixtures. The viscosity of reaction mixtures containing extraction buffer and substrate (EB) remained unchanged (see Table 3.2.2a).

This experiment showed that crude extracts of *Cuscuta* stem caused solutions of PGURA to become progressively solid with time. When the protein was denatured by heat the crude extracts no longer had this effect. It was concluded that the crude extracts contained pectin esterase.

The latter two experiments have demonstrated that PE was present in coiled and uncoiled *Cuscuta* stem. Further experiments were now performed to determine how the activity of the enzyme changed with time during infection. This was done in order to see if changes in PE activity during the penetration of the host were associated with any particular morphological or structural changes.

3.2.3 The change in the activity of pectin esterase (PE) in extracts of the tight coiled regions of the parasite during the time from initial coiling to penetration

The aim of this experiment was to measure the change in PE activity with time from initial coiling to penetration.

Experimental

Twenty, 100mm stem apices of *Cuscuta campestris* were inoculated on to the petioles of emerging *Pelargonium* leaves on three consecutive days. Twenty four hours after inoculation each set of apices were checked to see if they had formed tight coils around the host. On the fourth day the 1, 2 and 3 days old coiled regions were harvested. During harvesting each tight coiled region was examined to see if penetration of the host had occurred. They were then excised with a sharp razor blade in the cold room and weighed

Table 3.2.2a. The change in viscosity with time of reaction mixtures described in Section 3.2.2. CX = crude extract, BCX = boiled crude extract, EB = extraction buffer, * = reaction mixture had become solid

replicate	reagent mixed with substrate	flow time measurement in seconds			
		0 hr	6hr	7hr	24hr
1	CX	11.0	9.5	*	*
2	CX	12.0	10.0	*	*
3	CX	10.0	*	*	*
1	BCX	11.5	11.5	11.5	11.5
2	BCR	11.0	11.0	11.0	11.0
3	BCR	12.0	12.0	11.5	11.5
1	EB	13.5	11.5	11.5	11.5
2	EB	10.5	10.5	10.5	10.5
3	EB	10.5	11.0	11.0	11.0

Table 3.2.2b. The protein concentrations in extracts prepared from uncoiled *Cuscuta* stems CX = crude extract CXB = boiled crude extract

Extract	Replicate	Prot. per 10µl sample	Protein (mg ml^{-1})
CX	1	19.08	1.908
CX	2	11.22	1.122
CX	3	17.04	1.704
CXB	1	25.10	2.510
CXB	2	18.88	1.888
CXB	3	21.22	2.122

on a Sartorius digital balance. Protein was extracted from 40mg samples from each group of coils by the freeze/thaw method (see section 5.I.2 of Ch2). The crude extracts were assayed for P.E. activity by the method described in section 6.5 of Ch2. Aliquots of the crude extract were also assayed for protein (see section 5.I.2 Ch2). P.E. activity was expressed on a fresh weight, per coil and a per mg protein basis and plotted against time.

Results

The number of tight coiled regions per 40 mg sample decreased with increasing age of the interaction (see Table 3.2.3a). It was shown in section I of this chapter that the length of the tight coiled region did not increase after the initial coiling. Therefore in this experiment as the number of coiled regions per 40mg sample was increasing, the mean fresh weight of the tight coiled regions must also have been increasing with time. The weight of the coiled regions of I day-old interactions was so low that there were only enough coiled regions to make up one sample. After three days I2.5% of the interactions had penetrated the host tissues.

The changes in enzyme activity - Table 3.2.3a The mean activity per ml of extract decreased between day I and 2 but then increased again by day 3. The increase in activity between the second and third days was significant at the 5% level. When activity was expressed on a per mg protein basis (see Table 3.2.3b) the pattern of change with time was similar to that per ml of extract. The change in activity between the second and third days was significant at the 5% level. When the PE activity was expressed on a per coil basis (see Table 3.2.3b) it was found to increase progressively with time. The change in activity between days 2 and 3 was significant at the 5% level.

The changes in protein concentration - Table 3.2.3a The mean protein concentration per mg fresh weight was the highest for I day-old coils, decreasing after 2 days, with a slight increase on the third day. When protein was calculated on a per coil basis, the mean protein content was found to progressively increase with time (Table 3.2.3a).

A decrease in protein per g fresh weight with time would suggest that:-

(a) That there was a net fall in the amount of protein

(b) The amount of protein present remained the same but other components were increasing in quantity.

(c) There was a net increase in the quantity of the protein but the proportion that this protein contributed to the fresh weight was decreasing.

Expressing the protein on a per coil basis (see Table 3.2.3a) showed (c) to be the most likely. The amount of protein per coil increased with time. The increase in PE activity per coil was not directly proportional to the increases in protein. A doubling in the PE

Table 3.2.3a The protein concentration and pectin esterase activities per ml of extract, per mg protein and per coiled region. A reaction mixture has an activity of one unit when the addition of 1ml of 0.02M alkali is required to maintain its pH at 7.5 during one hour.

Age of coiled region (days)	number of coiled region per sample	activity per ml of extract	protein per ml of extract (mg)	activity per mg protein	activity per coiled region
1	10	31.2	2.37	13.16	0.156
2	5	14.4		8.08	0.144
	5	10.4		5.20	0.104
	3	20.0		10.28	0.332
3	4	-	2.05	-	-
	4	33.6	2.24	15.00	0.420
	4	32.0	2.22	14.44	0.400
	4	24.0	1.79	13.40	0.300
	4	33.6	1.72	19.60	0.420

activity between days 2 and 3 was not paralleled with a doubling in the amount of protein (see Table 3.2.3). The change in activity of the enzyme was therefore not only due to an increase or decrease in the overall quantity of protein.

This experiment showed that in the first 3 days after inoculation there were increases in the fresh weight of coils, the amount of protein per coil and the amount of PE per coil. The increase in the PE activity was not directly proportional to the increase in protein.

The investigation of the change in PE activity with time was continued with an experiment covering a longer period.

3.2.4 The changes in pectin esterase (PE) activity in extracts of tight coiled regions of *Cuscuta campestris* , 2 to 8 days after inoculation

The aim of this experiment was to measure the changes in the activity of PE with the change in the age of the host/parasite interaction.

Experimental

The extracts of tight coiled regions of different ages which were used in this experiment were the same as those used in section 3.I.2 of this chapter. At intervals from 2-8 days after initial coiling 25µl aliquots of these crude extracts were assayed for PE activity as described in section 6.4 of Ch2. Aliquots of 25µl were mixed with 7.5ml of substrate. PE activity was expressed per ml of extract, per mg protein and per coil. Protein concentrations were presented in section 3.I.2. and can be seen in Fig 3.I.2b and c.

Activity per ml of extract (Fig 3.2.4a) The mean PE activity per ml of extract decreased between days 2 and 3 then increased between days 3 and 4, 4 and 5, 5 and 6 and 6 and 7 before falling again on day 8 (Fig. 3.2.4a). However only the change between days 6 and 7 and 7 and 8 were significant at the 5% level. The differences in mean PE activity per ml of extract between days 3 and 4 and 3 and 5 were not significant at the 5% level. By day 6 and 7 the mean activities per ml of extract were significantly greater than at day 3. Fig 3.2.4b shows the change in PE activity per mg protein with time. The mean PE activity increased between days 2 and 3, 3 and 4 and 4 and 5. There was a slight depression in mean activity between days 5 and 6 and then an increase in activity between days 6 and 7. Finally there was an increase between days 7 and 8. The change in activity between days 6 and 7 was significant at the 5% level.

Activity per mg protein (Fig 3.2.4b) The activity per mg of protein after 3 days was not significantly greater at the 5% level than that after 2 days. However the activity on days 4, 5, 6, 7 and 8 was significantly greater than that on day 2. On days 5, 7 and 8 the activity was significantly greater than that at 2 days at the l% level. It was concluded that the activity per mg protein increased as the coiled region became more mature, between 2 and 8 days.

Fig. 3.2.4a. The change in the mean pectin esterase (PE) activity per ml of extract of tight coiled regions of *Cuscuta campestris* between 2 and 8 days after inoculation onto *Pelargonium* petioles. (I) = standard error bars.

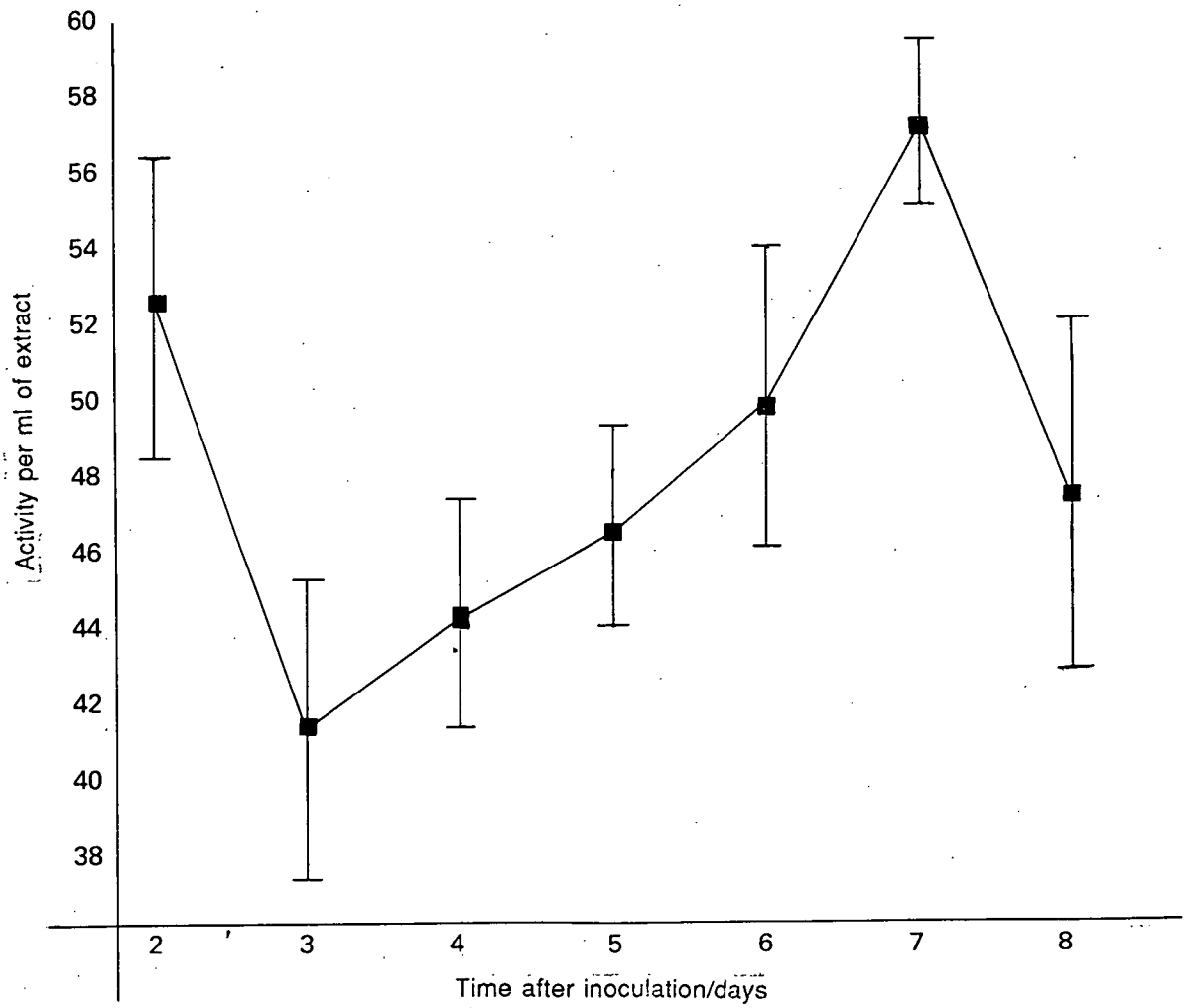


Fig. 3.24b. The change in the mean pectin esterase (PE) activity per mg of protein extracted from the tight coiled regions of *Cuscuta campestris* between 2 and 8 days after inoculation onto *Pelargonium petioles.* (⊥) = standard error bars.

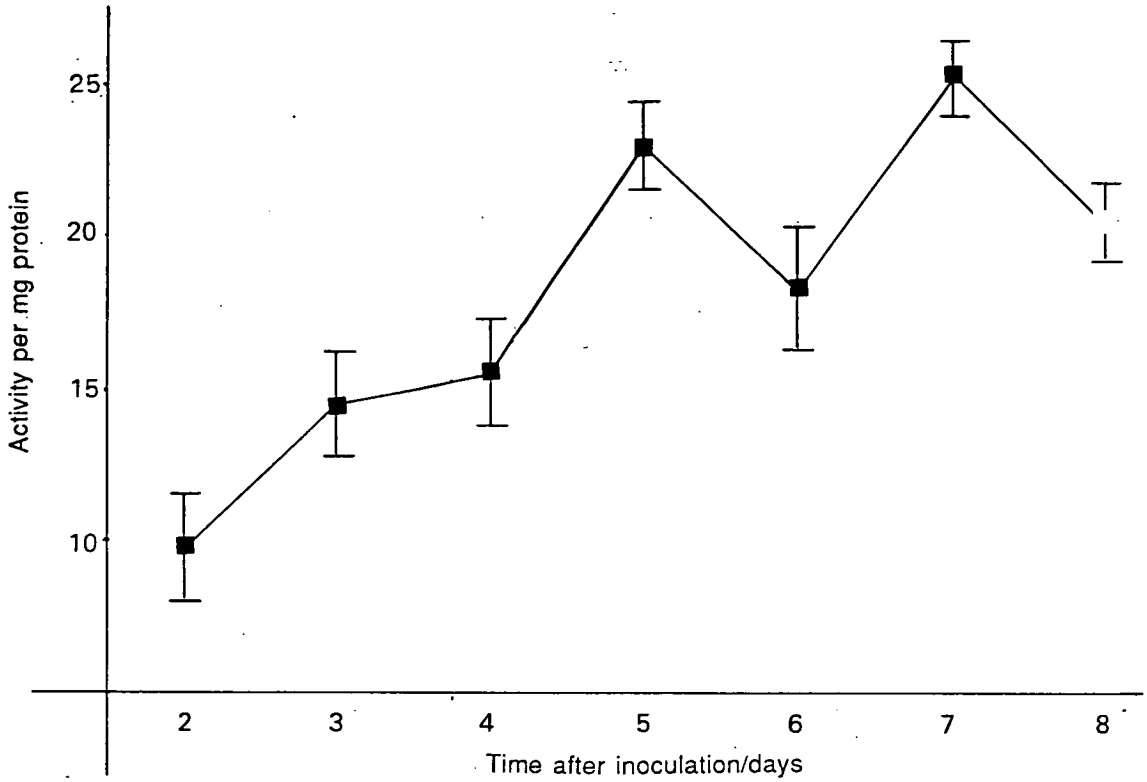
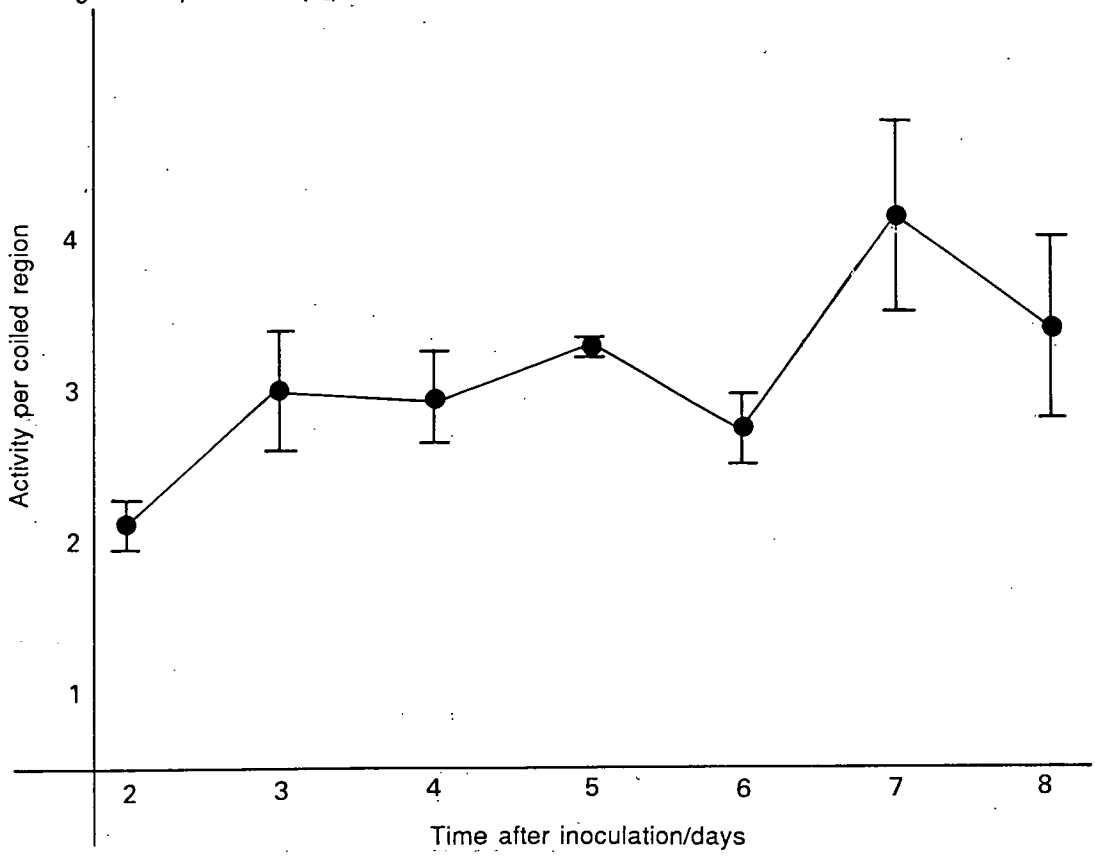


Fig. 3.2.4c. The change in the mean pectin esterase (PE) activity per coiled region for tight coiled regions of *Cuscuta campestris* between 2 and 8 days after inoculation onto *Pelargonium* petioles. (I) = standard error bars.



Activity per coil (Fig. 3.2.4c) The mean PE activity per coil increased between days 2 and 3, 3 and 4 and 4 and 5. The activity per coil then decreased before rising again between days 6 and 7, with a further decrease between days 7 and 8. The activity on days 4, 5, 6, 7 and 8 was significantly greater than that on day 2 at the 5% level. This showed that the activity in the coils was increasing as they matured. The activity on day 7 was significantly greater at the 5% level than that on day 6. An increase in PE activity per coil with time demonstrated that the enzyme was increasing in activity, and possibly quantity with the time coiled round the host.

This experiment showed that the PE activity per mg protein and per coil increased with time in culture. An increase in the activity per mg protein showed that the change in activity was to some degree independent of the change in protein concentration.

PE is just one enzyme known to be involved in cell wall breakdown which has been found at elevated levels in the parasitic situation. Another often found in association with PE is polygalacturonase (described in Ch1). A series of experiments was performed to see if this enzyme could be detected in the region of the host/parasite interaction between *Custcuta campestris* and *Pelargonium zonale* . These experiments are described below.

3.3.I The polygalacturonase (PGURASE) activity in extracts of penetrating *Cuscuta campestris* coils measured by a viscometric method

The aim of this experiment was to measure the (PGURASE) activity in coiled *Cuscuta* stem.

Experimental

The method used was that described in section 6.2 of Ch2. The assay was performed at pH 4.0, 4.5 and 5.0. This involved three separate experiments. For each experiment coiled *Cuscuta campestris* stem bearing penetrating haustoria was dissected away from excised *Pelargonium* petioles in the cold room. Protein was extracted from three, 0.2g samples of coiled tissues (approximately I0 tight coiled regions in 0.2g) by the freeze/thaw method using acetate extraction buffer (see section 5.I.2 of Ch2).

Following extraction the protein in the crude extracts was transferred to 0.05M acetate buffer at the pH required for that experiment (4.0, 4.5 or 5.0). The transfer was achieved by the spun column method (see section 5.2.I of Ch2). In each experiment all the components of the reaction mixtures were adjusted to the required pH for that experiment. The PGURASE activity was determined by measuring the change in viscosity with time of mixtures of protein and PGURA in 0.05M acetate at the appropriate pH (4.0, 4.5 or 5.0). Details of the procedure are described in section 6.2 of Ch2. Controls consisted of extraction buffer mixed with the substrate. The experiments were performed inside a constant temperature room. The flow times of the various reaction mixtures in each experiment were measured at the following times:

experiment number	pH	time in hours	temperature ^{o}C
1	5.0	0, 3.16 22.50	23
2	4.5	0, 6.16	22
3	4.0	0, 5.00	23

The flow times of the reation mixtures are recorded in Table 3.3.la, b and c.

The experiment performed at pH5

Table 3.3.la show the flow times for reaction mixtures in this experiment (pH5). After 3.16 hr the flow times of the reaction mixtures containing crude extract and substrate had not decreased more than 0.5 s. After 3 hr samples 1 and 2 gave the same flow time as at time zero. Sample 3 had a slightly longer flow time and samples 4 and 5 had flow times which had only decreased by 0.5 s compared to those at zero time. After 22.5 hr the flow times of samples 1, 3 and 4 were all greater than the flow times at time zero. The pipettes used to measure samples 2 and 5 had become blocked. Sample 6 which contained crude extract plus substrate became solid. This demonstrated that PE was present in the crude extracts. The controls which contained extraction buffer all showed slight increases in flow time over 22.5 hr. The degree of change in viscosity shown by the percentage change in the flow times was similar for experimental reaction mixtures and controls. It was therefore concluded that there was no measurable PGURASE activity in the reaction mixtures.

The experiment performed at pH4.5

Table 3.3.1 shows the flow times of reaction mixtures in experiment 2 (pH4.5). After 6.16 hr three of the samples (3, 4 and 5) had slightly faster flow times than they had at the start of the experiment. Sample 1 showed a slight increase in flow time while that for sample 2 stayed the same. The percentage change in flow time for the controls (samples 7-10) were similar to those for the experimental treatments. It was therefore concluded that there was no evidence of PGURASE activity in these reaction mixtures. Sample 7 became solid, demonstrating the presence of PE. A similar set of results were seen for the experiment carried out at pH4.0.

The experiment performed at pH4.0

The results for this experiment are seen in Table 3.3.lc. Four of the samples containing *Cuscuta* protein extract showed moderate decreases in viscosity after 5 hr (samples 1, 2, 3 and 4). The flow time of the fifth sample was the same as at the beginning of the experiment. The percentage change in the flow times of the controls (samples 7 - 10 Table 3.3lc) were similar to those for the experimental treatments. It was concluded that there was no evidence of PGURASE activity in this experiment. Sample 7 became solid

Table 3.3.1a The change in viscosity with time for reaction mixtures from section3.3.1 RM = reaction mixtures, P+S = *Cuscuta* extract plus substrate, CE+S = crude extract plus substrate, EB+S = extraction buffer plus substrate * = the reaction mixture had become solid.

sample	RM	pH	Flow time in seconds			% change in flow time
			0 hr	3.16 hr	22.5 hr	after 22.5 hr
1	P+S	5	20.5	20.5	21.0	+ 2.43
2	P+S	5	21.5	21.5	-	-
3	P+S	5	20.0	21.5	24.25	+ 12.12
4	P+S	5	22.0	21.5	27.25	+ 23.80
5	P+S	5	18.0	17.5	-	-
6	CE+S	5	18.0	*	*	*
7	EB+S	5	23.0	22.5	25.25	+ 11.96
8	EB+S	5	19.5	19.0	21.5	+ 10.25
9	EB+S	5	20.5	20.75	24.5	+ 19.51
10	EB+S	5	22.5	22.25	22.75	+ 1.11

Table 3.3.1b The change in viscosity of reaction mixtures from experiment 'b' of section 3.3.1. Abbreviation are as in table 3.3.1a

sample	RM	pH	Flow time in seconds		Percentage change in flow time after 6.16 hr
			0 hr	6.16 hr	
1	P+S	4.5	20.5	22.0	+ 7.3
2	P+S	4.5	19.0	19.0	0
3	P+S	4.5	21.0	20.5	- 2.3
4	P+S	4.5	18.5	17.5	- 5.4
5	P+S	4.5	22.0	20.5	- 6.8
6	CE+S	4.5	21.0	*	*
7	CE+S	4.5	20.5	*	*
8	EB+S	4.5	17.5	16.0	- 8.6
9	EB+S	4.5	20.5	19.5	- 4.9
10	EB+S	4.5	19.0	18.5	- 2.6

Table 3.3.Ic The change in viscosity of reaction mixtures from experiment 'c' of section 3.3.1. Abbreviations are as in table 3.3.1a

sample	RM	pH	Flow times in seconds		Percentage change in flow time after 5 hr
			0 hr	5 hr	
1	P+S	4.0	18.0	17.5	- 2.78
2	P+S	4.0	19.0	18.75	- 1.32
3	P+S	4.0	16.5	15.75	- 4.58
4	P+S	4.0	15.0	14.25	- 5.00
5	P+S	4.0	19.0	19.0	- 0.00
6	CE+S	4.0	19.0	*	*
7	EB+S	4.0	17.5	17.75	+ 1.40
8	EB+S	4.0	20.0	19.0	- 5.00
9	EB+S	4.0	17.5	17.0	- 2.94
10	EB+S	4.0	24.0	22.0	- 8.30

demonstrating the presence of PE.

These experiments showed that PGURASE could not be detected by this viscometric method. There are several possible reasons for inability to detect the enzyme. It may have been that the enzyme was present in the tissues but at a very low concentration. Secondly, this method may not be suitable for detecting the type of PGURASE present. This method is designed for detecting endopolygalcturonases. Another type of assay was therefore used. This method has been used by Nagar *et al.* (I984) who claimed to measure PGURASE in stems of *Cuscuta reflexa.* The experiment using this method is described below.

3.3.2 The polygalacturonase (PGURASE) activity in extracts of penetrating, coiled and uncoiled *Cuscuta campestris* stem measured by a reducing sugar method

In this section the results of two experiments are reported. The aim of these experiments was to measure the activity of PGURASE in the coiled and uncoiled parasite stems.

Experimental

The PGURASE activity of the enzyme was measured by the reducing sugar method described in section 6.I of Ch2.

In the first experiment protein was extracted from twenty-four 40mg samples of tight coiled *Cuscuta campestris* stem by the freeze/thaw method, using acetate extraction buffer. In the second experiment protein was extracted from five, 0.2g samples of uncoiled *Cuscuta campestris* stem (internode tissues at least I0mm from the apex or any node) by the freeze/thaw method.

In the first experiment the crude extracts were pooled together and then divided into two portions. The first portion was passed through a spun column of Sephadex to remove salts and small molecules as described in section 4.2.I of Ch.2. Aliquots of the purified extract and the untreated second portion were used in the reaction mixtures of the assay procedure. In the second experiment aliquots of crude extract only were used in the reaction mixtures of the assay. The constituents of the reaction mixtures in the two experiments were as follows:

REACTION MIXTURES (RM)	1	2	3	4	5	6	7	8	9
crude extract	62.50	-	-	-	62.50	-	-	-	-
desalted protein extract	-	62.50	-	-	-	62.50	-	-	-
1% PGURA in 0.05M acetate buffer pH5	31.25	31.25	31.25	31.25	-	-	-	-	-
0.05M acetate buffer pH5	18.75	18.75	18.75	81.25	50.0	50.0	50.0	112.5	125.0
sodium dithionite in 0.05M acetate buffer pH5	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	-

Experiment 2

REACTION MIXTURES	I	2	3	4	5	6
crude extract	62.50	62.50	-	-	-	-
acetate extraction buffer	-	-	62.50	62.50	-	-
ImM sodium dithionite in acetate buffer pH5	I2.50	I2.50	I2.50	I2.50	I2.50	-
I% PGURA in 0.05M acetate extraction buffer pH5	3I.25	-	-	3I.25	-	-
0.05M acetate buffer	I8.75	50.00	I8.75	50.00	II2.50	I25.00

In the first experiment six replicates of each reaction mixture were made up; three were incubated at 25°C and three at 37°C for 4 hrs. In the second experiment three replicates of each reaction mixture were made up and incubated at 37°C for 5 hrs. Following incubation the reducing power in each reaction mixture was estimated by the method described in section 6.5 of Ch2.

The reducing power in the different reaction mixtures

The absorbances of RM 6, 8 and 9 in the first experiment (Table 3.3.2b) were close to zero. This demonstrated that acetate buffer, sodium dithionite solutions and the desalted protein extract contained no reducing power. The extraction buffer did contain some reducing power (see RM 7 Table 3.3.2b). The reducing power of incubated crude extract was greater than that for extraction buffer and substrate (see RMI and 3). The reducing power in reaction mixtures containing crude extract but no substrate was as high as those which contained substrate (see RM5 experiment I). The reaction mixture containing mixtures of desalted protein and PUGRA (RM2) had reducing power values less than those for PUGRA alone (RM4).

If there was PGURASE activity in the crude extract, reducing power would have been generated during incubation. Under these circumstances the measured reducing power would be expected to be greater than the sum of reaction mixtures 5 and 4. This was not the case in this experiment. It was therefore concluded that there was no measurable PGURASE activity present in the crude extract. The amount of reducing power present in RM2 of the first experiment would suggest that there was no PGURASE or very little activity in the desalted protein extract. If PGURASE was present in the purified extract the reducing power in the mixture of desalted protein and I% PUGRA would have been expected to have been greater than that for I% PUGRA alone.

The cells of *Cuscuta campestris* contain large amounts of visible carbohydrate storage material. This may have been the source of the reducing power found in the crude extract.

In the second experiment the reducing power due to the dithionite and acetate was found to be zero (RM8 and 7 Table 3.3.2a). The reducing power due to the PGURA was calculated by subtracting the values obtained for RM5 from those of RM4 (0.I56). Extraction buffer was found to contain reducing power (see RM4 Table 3.3.2a). The reducing power of the crude extract (RM2) was greater than that of the extraction buffer (found by subtracting the mean value for RM4 from that for RM2). The reducing power of RM2 containing crude extract but no substrate was similar to that containing crude extract and substrate (RMI). The reducing power of the RMI containing crude extract and PGURA was less than the reducing power due to the crude extract (RM2) plus calculated value for PGURA (RM4 minus RM3). This would suggest that there was no reducing power generated during the period of incubation and therefore no PGURASE present in the crude extract.

Table 3.3.2a The absorbances values at 520nm of reaction mixtures 1-6 of experiment 2 in section 3.3.2a Rm = reaction mixture

RM	Absorbance at 520 nm			mean absorbance
	1	2	3	
.1	1.950	1.956	2.004	1.970
2	1.939	1.969	1.848	1.919
3	1.200	1.116	-	1.158
4	1.006	0.998	-	1.002
5	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000

Table 3.3.2b The absorbance at 520nm of reaction mixtures 1-9 from experiment 1 of section 3.3.2 Rm = reaction mixture.

RM	incubation temperature (°C)	Absorbance at 520nm 1	2	3	mean absorbance
1	25	1.752	1.708	1.752	1.737
2	25	0.234	0.210	0.211	0.218
3	25	1.218	1.228	1.243	1.230
4	25	0.429	0.373	0.383	0.395
5	25	1.667	1.716	1.707	1.697
6	25	0.000	0.002	0.000	0.000
7	25	0.912	0.977	0.934	0.941
8	25	0.000	0.000	0.000	0.000
9	25	0.000	0.000	0.000	0.000
1	37	1.641	1.709	1.650	1.667
2	37	0.220	0.227	0.275	0.241
3	37	1.048	1.131	1.069	1.083
4	37	0.455	0.447	-	0.451
5	37	1.657	1.740	1.623	1.673
6	37	0.000	0.000	0.000	0.000
7	37	0.928	0.892	0.894	0.905
8	37	0.000	0.000	0.000	0.000
9	37	0.000	0.000	0.000	0.000

Table 3.3.2c The absorbance at 520nm of standard galacturonic acid solutions from experiments 1 and 2 of section 3.3.2. G.A. = galacturonic acid, concentration

conc of G.A. in mg/ml	Absorbance at 520nm 1	2	3	mean absorbance
expt 1				
0.1	0.274	0.286	0.283	0.281
0.2	0.625	0.597	0.567	0.596
0.3	0.929	0.955	0.935	0.940
0.4	1.282	1.168	1.219	1.223
0.5	1.366	1.397	1.365	1.376
expt 2				
0.1	0.203	0.199	0.194	0.199
0.2	0.456	0.489	0.468	0.471
0.3	0.679	0.703	0.743	0.707
0.4	0.914	0.908	0.905	0.909
0.5	1.126	1.124	1.129	1.126

The reducing power present in the crude extract may have come from carbohydrate extracted from the stem of *Cuscuta.*

This experiment showed that the PGURASE activity was not measurable by the method and was likely to be absent or very low in the tissues of coiled and uncoiled *Cuscuta campestris*

Assays for PGURASE were also performed on host plant material. It was concluded from section 3.I.5 that the CE activity in *Pelargonium* petiole tissues was absent or very low. The protein concentration per unit weight of petiole was lower than that in the parasite. These findings were taken into consideration in planning the PGURASE assays. Extracts were concentrated before being assayed to increase the amount of protein in individual aliquots. An experiment measuring PGURASE in *Pelargonium* petioles is described below:

3.3.3 The polygalacturonase (PGURASE) activity in extracts of uninfected *Pelargonium* petiole tissues

The aim of this experiment was to measure the PGURASE activity in *Pelargonium* petioles.

Protein was extracted from l0g of petioles excised from emerging *Pelargonium* leaves by the grinding method described in section 5.1.1 Ch2. A 0.5ml sample was removed from the crude extract before it was divided into four portions. Each portion was dialysed overnight against 2l of 0.05M acetate buffer in the cold room in order to remove salt. Portion I was dialysed against pH 5 buffer, portion 2 pH 4.5, portion 3 pH 4.0 and portion 4 pH 3.5. Following dialysis extracts were concentrated in a refrigerated Speed Vac concentrator for 2 hrs reducing their volumes by approximately 50% (800µl aliquots were rotated under vacuum in open Eppendorf capsules). The dialysed, concentrated extracts were then stored on ice in the cold room before being used in a reducing sugar assay for PGURASE.

The assay method used was exactly as described in section 6.5 of Ch2. Twenty- three reaction mixtures were set up, bearing the following codes: Ia, Ib, 2, 3a, 3b, 4, 5, 6, 7a, 7b, 8, 9, I0, IIa, IIb, I2, I3, I4, I5a, I5b, I6, I7 and I8. RM IA, IB and 2 were exactly as in section 6.5 of Ch2. The rest of the RM could be divided into 4 groups as follows:

		RM					
		a	b	c	d	e	
Groups							
(I)	(	3A	3B	4	5	6	)
(2)	(	7A	7B	8	9	I0	)
(3)	(	IIA	IIB	I2	I3	I4	)
(4)	(	I5A	I5B	I6	I7	I8	)

Each group of RMs were the same except for their pH values. Groups I, 2,3 and 4 had values of 5.0, 4.5, 4.0 and 3.5 respectively. RMs in the same columns (a,b,c,d ande) in the table contained the same constituents. These constituents were as follows:

	RM				
CONSTITUENT	a	b	c	d	e
Crude Protein Extract	0	0	0	0	0
Desalted Protein Extract	62.50	62.50	0	0	0
0.05M acetate buffer	I8.75	18.75	8I.25	II2.50	I25.00
ImM Sodium dithionite in 0.05M acetate buffer	I2.50	I2.50	I2.50	I2.50	0
I% PGURA in 0.05M acetate buffer	3I.25	3I.25	3I.25	0	0

The buffer, sodium dithionite and PGURA components of the 'B' reaction mixtures were mixed together and then incubated for 4hr at 37^{o}C before the protein extracts were added. All the other reaction mixtures were completely made up before incubation. For the 'B'

Table 3.3.3a The Absorbance at 520nm of reaction mixtures of different pHs from the experiment in section 2.3.3.

RM	pH	absorbance at 520nm 1	2	3	mean absorbance	Difference between incubated and unincubated
1a	5	2.287	2.302	2.303	2.297	
1b	5	2.323	2.314	-	2.318	
2	5	1.876	1.819	1.889	1.861	
3a	5	1.713	1.629	-	1.671)	
3b	5	1.500	1.475	-	1.487)	0.183
4	5	1.301	1.325	1.334	1.320	
5	5	0.000	0.000	0.000	0.000	
6	5	0.000	0.000	0.000	0.000	
7a	4.5	1.808	1.810	1.807	1.808)	
7b	4.5	1.724	1.602	1.743	1.689)	0.119
8	4.5	0.297	0.281	0.342	0.307	
9	4.5	0.000	0.000	0.000	0.000	
10	4.5	0.000	0.000	0.000	0.000	
11a	4.0	2.028	2.010	2.025	2.021)	
11b	4.0	1.946	1.928	-	1.937)	0.084
12	4.0	0.440	0.361	0.346	0.382	
13	4.0	0.000	0.000	0.000	0.000	
14	4.0	0.000	0.000	0.000	0.000	
15a	3.5	2.057	2.065	-	2.061)	
15b	3.5	1.998	-	-	1.998)	0.063
16	3.5	0.362	0.318	-	0.340	
17	3.5	0.000	0.000	0.000	0.000	
18	3.5	0.000	0.000	0.000	0.000	

Table 3.3.3b The polygalacturonase activity of concentrated protein extracts (section 2.3.3) in mg of galactoronic acid per ml per hr

Extract	Activity
pH 5.0	1.8
pH 4.5	1.4
pH 4.0	1.0
pH 3.5	0.9

reaction mixtures the amount of reducing power was estimated immediately after addition of the protein extract. All other reaction mixtures were assayed immediately after incubation. The procedure for estimating the reducing power present in each reaction mixture is described in section 6.5 of Ch2. A method for translating this reducing power into units of activity is aiso given in that section.

Results- reducing power in the reaction mixtures

No reducing power was found in the acetate buffer or mixtures of buffer and sodium dithionite after incubation (RM5 and 6, 9 and I0, I3 and I4 and I7 and I8 Table 3.3.3a). Reducing power was found in solutions of extraction buffer and of I% PUGRA. Reaction mixtures containing crude extract and substrate which were incubated (RMIa) had similar reducing power to those which were not incubated (RMIb). No measurable reducing power was generated as a result of incubation of 4hr. Reaction mixtures containing purified, concentrated extract and substrate (RM 3a, 7a, IIa and I5a) which were incubated complete (with all their constituents), had larger reducing power values than identical reaction mixtures incubated without the substrate (RM 3b, 7b, IIb and I5b). The unincubated crude extract (RMIb) contained more reducing power than that of extraction buffer (RMIb-RM2). As this reducing power could not have been generated as a result of incubation it must have been extracted from the petiole tissues. Petioles contain large amounts of non-growing parenchymatous cells. The reducing power in the crude extract may have resulted from carbohydrate stored in these cells.

The larger reducing power present in reaction mixtures which had been incubated with the substrate would suggest that PGURASE was present in the extracts. By referring to standard curves calculated for galacturonic acid (see Appendix 3.3.3.3) values for the PGURASE activity were calculated in mg of galacturonic acid per ml of extract per hr. The calculated values are shown in Table 3.3.3b The largest value was obtained for the pH5 concentrated extract, with decreasing activities in the pH4.5, 4.0 and 3.5 extracts. Unfortunately the protein content in each extract was unknown, so values of the activity per mg protein were not available. Therefore it was not possible to determine the optimum pH of the assay. The lack of difference between reaction mixtures IA and IB was probably due to the enzyme being in too low a concentration in the crude extract to be detectable with this assay procedure. Longer incubation times may have revealed differences in the two types of reaction mixture.

This experiment showed that PGURASE could be detected at a low level in petioles of *Pelargonium.*

Section 3.3 showed that PGURASE activity was very low in extracts of *Pelargonium* petioles and apparently absent from extracts of coiled or uncoiled *Cuscuta campestris.*

Chapter 4

Chapter 4 - THE DISCUSSION

This discussion is divided into four main sections:-

(1) A discussion of the development of the *in vitro* system.

(2) A discussion of this host/parasite interaction at the organismal, cellular and molecular levels from experiments performed on both *in vivo* and *in vitro* material.

(3) A discussion of the success of *Cuscuta* as a parasite, paying particular attention to its invasion of *Pelargonium.* Combined with this are some suggestions of possible mechanisms the plant uses to overcome host resistance.

(4) Possible future work

Section 1 The development of the *In vitro* system

1.1 A discussion of some of the properties of excised *Cuscuta campestris* shoot tips which were revealed during the development of the *in vitro* system

1.1.1. The ability of shoot tips to extend when excised from the rest of the plant

The young seedling is not the only effective infective agent. Excised shoot tips, comprising an intact apex plus a length of stem, are able to mobilise their stored nutrients (seen in cells as granules see section 1.2 of Ch3) and increase in length. This extension may represent a search for a new host plant. The faster extension rate observed with longer (40mm) shoot tips may be because of a larger nutrient supply in the longer tips The decrease in the rate of extension of shoot tips with time was presumably due to a decrease in the amount of stored reserves available to fuel growth. The extension rate of the longer shoot tips took longer to slow down. This is consistent with them having larger food reserves. The decline in the rate of extension did not begin until more than 48hr after excision for 40mm tips implying that the supply of endogenous nutrients did not become limiting until that point. This gives some indication of how long shoot tips can survive on their own resources If lack of nutrient begins to limit extension after 48hr it is also likely to limit the shoot tips ability to coil around a host plant as this process involves growth. Therefore it is important that 30 and 40mm shoot tips make contact with a host within 32 and 48hr respectively before nutrients become limiting. As these shoot tips only double in length in 48 hr when stored in water it is also important that the host plant is only a short distance away.

Jacob *et al*. (1986) noticed that excised shoot tips of *Cuscuta reflexa* increase in

length. This suggests that the extension of excised shoot tips described here is a general feature of *Cuscuta* plants. Most other work involving excised *Cuscuta* shoot tips concentrates on *in vitro* work, where sterile shoot tips are grown in sterile liquid culture medium. (eg Maheshwari *et al.* 1980)

1.1.2. The dependence of the ability to infect on the shoot tip length

The shrivelling of the cut end and the swelling of the tight coiled region of a shoot tip which is infecting a host plant strongly suggests that all the available nutrients are being used to making invasion a success. The growth of young invasive *Cuscuta* plant parts at the expense of older parts has been frequently observed by those working with *Cuscuta* (Jacob *et al.* 1986). The parasite apparently has the ability to concentrate all the available nutrient within a shoot tip into a particular area. Therefore the longer the excised shoot tip the more nutrients are available to contribute to the invasion of the host.

In order to infect a host plant a shoot tip must be able to perceive a tactile stimulus and contain sufficient food reserves and moisture to develop tight coils and invasive structures (haustoria). The inability of the shorter shoot tips described in sections 2.1.2 and 2.1.3 of Ch3 to coil may have been a result of a deficiency in either or both of these factors. Shoot tips 10mm in length might be expected to have insufficient food reserves and moisture to sustain the growth required to infect. Assuming that the petiole diameter is approximately 2mm (Section 2.3.2. Ch3) and the circumference approximately 6.3mm ($2\pi r$) the shoot tip must extend more than half its length again to form a single turn of a coil around the host. Longer 30mm shoot tips placed in water only increased by two thirds in 32hr. Fritshe *et al.* (1958) showed that the extreme tip is the region of *Cuscuta* stems which is most sensitive to tactile stimulae. It is therefore unlikely that the reason for 10mm shoot tips not coiling was an inability to receive the necessary stimulae. It is more probable that the amount of available nutrients were insufficient to allow the shoot tips to respond to such stimulae once they had been recieved.

An increase in the coiling frequency as shoot tips become longer between 10mm and 50mm (see section 2.1.2 of Ch3) suggests that there is something about the longer shoot tips which makes them more likely to infect a host plant. Successful infection by a 20mm shoot tip shows that it is possible for shorter tips.

Twenty millimeters may however be approaching the minimum length at which infection can proceed if there is no delay in between inoculation and coiling. It would be interesting to see how 20mm shoot tips extended when excised and placed in water. It may be that ability to coil and ability to extend are both closely linked to the nutrient supply. Shoot tips 20mm and less may show a similar reluctance to extend as they did to coil when inoculated.

The orientation of the terminal 5-10 mm of inoculated shoot tips was very similar

regardless of length. The failure of the shorter tips to coil is therefore unlikely to be due to differences in the procedure used.

1.1.3. The importance of moisture in infection of *Cuscuta* shoot tips

It has been shown that the number of stages of infection achieved by inoculated shoot tips was related to length in the range 30 to 50 mm (see section 2.1.3 Ch 3). Without coiling infection cannot take place. However the formation of tight coils does not guarantee that all the subsequent stages will be completed. It seems sensible to suggest that shorter shoot tips cannot complete infection because their endogenous reserves become exhausted.

The observed increase in the frequency of successful infections for 30 to 50mm shoot tips which were stored in water prior to inoculation (see section 2.1.3 Ch3) suggests that an adequate supply of moisture was a more critical factor than the amount of energy reserves. While stored in water the energy reserves of shoot tips decrease due to continued respiration and extension growth. However as a result of this extension growth their water content increases. An increase in water content caused by extension growth increases the ability of 30 to 50mm shoot tips to infect despite the decrease in the energy reserves. It would seem therefore that moisture is a more limting factor than the amount of stored nutrient in determining the infectivity of shoot tips of this length.

Another possible reason for the increased success of shoot tips stored in water could be that the treatment washes out an inhibitory factor which is released when tips are excised and which limits their ability to coil and infect. However, freshly excised 60 mm shoot tips do not infect any less successfully than 60 mm shoot tips stored in water. It is therefore unlikely that an inhibitory factor is responsible for the differences in the infectivity of shorter tips which were directly inoculated or first stored in water. For 60mm shoot tips storage in water increases the moisture content but does not appear to increase their infectivity. The most probable reason is that shoot tips this length already contain all the moisture necessary to mount an infection.

1.1.4. The time taken to achieve various stages of infection by shoot tips of different lengths

The decrease in the time taken to reach each stage of infection with increasing length of inoculum was not statistically significant (see section 2.1.3 Ch3) and therefore will not be discussed further.

1.1.5. The response of excised shoot tips to unidirectional light

The growth of apices of *Cuscuta* seedlings towards a light source is likely to be a general characteristic of the parasite as it is for other Angiosperm plants. Growth

towards a light source is an advantage for a seedling emerging from the soil. In addition it is important for seedlings to grow upwards into the region populated with the stems of potential hosts. Spisar (1910) has also shown that *Cuscuta* is positively phototrophic. This in combination with a strong negative geotropism (Zeitz 1954) and its circumnutating growth pattern must ensure that a host is found.

1.1.6. Factors influencing the development of haustorial feeding organs

When shoot tips form tight coils in the air, prehaustoria are seen to develop. However, in contrast to those which form on coils which tightly grip the host, they do not differentiate distinct haustorial organs within them. This suggests that an adjacent host is necessary for haustoria to develop. A tactile and/or chemical signal may control the development of this organ. The experiment when sterile shoot tips successfully coiled around bamboo canes in a sterile , controlled environment (see section 2.4.1 Ch3) showed that coiling was possible using the experimental procedure which had been designed. It also showed that a haustorium could be stimulated to develop in the absence of a living plant. As the cane was of plant origin it cannot be eliminated that the development of the haustoria was because the parasite could perceive this. Nevertheless the results suggest that a tactile stimulus or physical pressure may influence haustorial development even in the absence of an appropriate host. This idea is supported by Dean (1937) who also suggested that the mechanical pressure between host and parasite was responsible for the development of haustoria. Tsivion (1978a) claimed that haustorial development and emergence is controlled by cytokinins and not by mechanical pressure. He performed experiments on string supports finding that haustoria did not develop unless a cytokinin was added to the string. String is not a very dense material and might not be expected to cause any build up of pressure between the parasite and itself. In a later publication Tsivion (1978b) showed that haustoria could develop in the absence of applied cytokinin when stems were induced to coil around denser non-porous parafilm. He then suggested that cytokinin was still responsible for haustorial development and that it was generated by the pressure induced death of cells at the parasite/parafilm interface. Other work therefore is found to be consistent with the evidence collected here, pressure is likely to be an important factor in haustorial development.

Section 1.2. The effect of culturing and the *in vitro* procedure on the parasite

1.2.1. The ability of parasite to shoot tips to grow and assimilate nutrient medium in culture

The changes in length, fresh weight and dry weight are considered separately.

The change in length: The observation that sterile shoot tips increase in length when only supplied with water (see section 2.2.1 Ch3) is consistent with earlier work *in vivo* (section 2.1.1 of Ch3). However, in contrast to the freshly excised 30mm and 40mm shoot tips described in section 2.1.1 of Ch3, the response is not consistent. There are several possible reasons why excised shoot tips did not extend when they were cultured in water:

(1) The growing apices could have become damaged

(a) Physically as a result of the manipulations involved during sterilisation and the preparations for culture.

(b) During the sterilisation the delicate apical regions could have been damaged chemically, by the hypochlorite (Na OCl) preventing any further extension.

(2) Certain shoot tips could have entered some dormant state during which extension does not occur. When *Cuscuta* plants approach flowering, the shoot tips do not extend at such a rapid rate and many branches do not give rise to new shoots but to flowers instead. Some of the shoot tips chosen may have been in a transition, not yet obvious from their morphology, between vegetative and flowering states.

The greater increases in the mean lengths of shoot tips cultured with liquid nutrient medium compared with those cultured in water between 0 and 3 days suggest that the former had a stimulatory effect on extension. The similarity in the changes in the mean extension of shoot tips cultured with or without GA_3 shows there was no extra stimulatory effect of including GA_3 in the medium. The growth substance did not have its usual affect of promoting growth (Paleg 1965).

The apparent falls in the mean shoot tip lengths of shoot tips which are cultured with basal nutrient medium with or without GA3 (see section 2.2.1 Ch3) shows that these media were unable to overcome whatever seems to prevent some excised shoot tips from extending when they were cultured in this way. It is important to discover what the factor is which causes some shoot tips not to extend when cultured, as they may be equally unable to coil and infect.

The increases in length of excised *Cuscuta* shoot tips cultured by Maheshwari *et al.* (1980) was very similar to those described in section 2.2.1 of Ch3 under similar conditions. However, they did not find that shoot tips varied so much in response to culture. The sterilisation procedure used by Maheshwari *et al.* (1980) was apparently more thorough than the one used here, indicating failure of some tips to extend was probably not attributable to damage during sterilisation.

In contrast with the results presented here Maheshwari *et al.* 1980 found that extension of shoot tips cultured with GA_3 was significantly greater than for shoot tips cultured without it. The cessation of extension for shoot tips cultured without GA_3 after

a short period of growth was not substantiated here. The differences between these findings and those of Maheshwari *et al.* 1980 may be due to their use of a different *Cuscuta* species.

The change in fresh weight: The pattern of change in the mean fresh weight with time was very similar to that for the change in mean length. This shows that the change in fresh weight of shoot tips is closely correlated with the change in length, this is not unexpected in a growing structure of more or less constant cross section and density.

Changes in dry weight: The decrease in the mean dry weight for shoot tips cultured in water only is what would be expected for a non-photosynthetic living organism having no input of nutrients but continuing to respire. The rise in the mean dry weight of shoot tips cultured with basal medium plus GA_3 showed that they were assimilating nutrients. The increase in the mean dry weight of shoot tips which were not extending suggest they were assimilating nutrients and putting them into storage. The increase in mean dry weights of shoot tips which were not increasing in length shows that these shoot tips were still alive. If shoot tips were not extending because they were damaged in some way these increases in dry weight show that the rest of the tissues were functioning properly. Maheshwari *et al* (1980) did not record changes in the fresh and dry weight of cultured Cuscuta shoots with time in culture, therefore no comparisons can be made with their work.

The very close similarity in the mean dry weights of shoot tips cultured for 14 days with or without GA_3 showed that this growth substance had no additional stimulatory effect on the rate of assimilation of exogenous nutrients.

This experiment may best be repeated with more replicates in order to establish that the lack of extension of some shoot tips was as common as it appeared. The effect of the sterilisation procedure on the ability to extend should also be further investigated. This could be done by varying the concentration and time exposed to Na OCl.

This ability of excised shoot tips to assimilate nutrients supplied in the culture medium is clearly a very important in the development of the *in vitro* system. It may be desirable to incorporate radioactive labels into the parasite *in vitro* by supplying them in the culture medium. A radioactively labelled parasite is likely to be a useful tool in experiments where it is desirable to determine the origin of the various components at the host/parasite interface. This has already been shown to be a useful method by Fer and Capdepon (1985). Incorporating substantial radioactive label into the parasite may take several days. The dry weight of shoot tips was shown to take seven days to double. The usefulness of the technique will depend upon the ability of cultured shoot tips to infect being retained over the incorporation period. Ability to infect is certainly retained after 4 days in culture as shown in section 2.2.2 of Ch3. In the next section the effect of the

culturing procedure on the ability to infect is discussed.

1.2.2. The effect of sterilisation and culturing on the ability to infect

The experiment which examined the effect of the culture procedure on the ability to coil and infect (see section 2.2.2 Ch3) showed that ability to infect *in vivo* was not reduced by culturing shoot tips for 3 days. The larger frequencies of successful infections for 3 and 1 day cultured shoot tips compared to freshly excised shoot tips suggests that the sterilisation procedure did not impair their ability to infect. The finding that all the shoot tips which were cultured extended with time is in contrast with the results of the experiment in section 2.2.1 Ch3. The sterilisation procedure used in the experiment described in section 2.2.2 Ch3 was the same as that used in the experiment in section 2.2.l of Ch.3. This suggests that the sterilisation procedure was not the reason why some shoot tips failed to extend in section 2.2.1. However the physical manipulations involved in the two experiments were different. Damage caused during orientating shoot tips in small tubes at the bottom of long narrow specimen tubes (see section 2.2.1 Ch3) may be the reason for lack of extension of certain shoot tips in that experiment. Another factor which can explain the difference in the two experiments is that the experiment described in section 2.2.2 was performed at a later stage than that in section 2.2.1. It may be that the increased experience in manipulating parasite shoot tips between the two experiments meant that less damage was caused to the plant material in the later one.

The impact of prolonged culture on the ability of excised shoot tips to infect cannot be predicted from the experiment described in section 2.2.2 Ch3. The shoot tips which performed least well in the experiment were those cultured for the longest time (4 days). A decrease in the ability of shoot tips to infect *in vivo* following increasing time in culture may be caused by the *in vitro* environment. The extremely high humidity inside plant containers may cause tissues to become very sensitive under *in vivo* conditions rather like plants which have been propagated *in vitro* and which require a 'weaning' period when being brought out into the field (Pillae and Hilderbrandt l968) during which they adjust to the new environment. If this was so it might be expected that a decrease in infectivity would not be experienced when cultured shoot tips were inoculated *in vitro.*

1.2.3. The frequency of coiling *in vitro*

The reaction of the sterile cultured parasite to the position of a light source (see section 2.4.1) and the charged surface of containers (see section 2.4) showed how important it was to carefully regulate the environment in which the host and parasite were to interact. Other attempts to infect sterile host plant material with sterile parasite (Geartner 1950) were not successful. Geartner (1950) found that the parasite grew towards and adhered to the container walls instead of coiling around the host. Perhaps a

more detailed study of the influences of the external environment on the growing parasite apex may have led to the success which has been achieved here.

Even when the influences of electrical charge on the container surface and directional light were removed the frequencies of coiling *in vitro* were very much lower than those *in vivo*. The following reasons for this are suggested:

(I) Other environmental factors were inhibiting the interaction.

(2) The apical regions were damaged

(a) during sterilisation

(b) during physical manipulations while being prepared for inoculation.

(3) The difference in the inoculation procedures used *in vitro* compared to that *in vivo*

(4) The extremely high humidity inside the containers may in some way have inhibited the coiling of the parasite.

Fritshe *et al.* (I958) described the apex of *Cuscuta europaea* shoot tips which is the region that is most sensitive to tactile stimulae from host plants. The surface of this region was said to be 'sticky' and this property aided the infection of a host by increasing the chances of the parasite staying in contact with the host in the early stages. When shoot tips are inoculated *in vitro* it may be that humidity or some other environmental factor interferes with this sticky layer decreasing their ability to coil. The temperature is unlikely to have been too high inside the plant containers as *Cuscuta campestris* is common in countries with extremely high summer temperatures. (Ashton and Santana I976).

It is possible that the reduced coiling frequency *in vitro* compared to that *in vivo* is due to damage by sterilisation with NaOCl. However, the results of the experiment which examined the effect of the culture procedure on the ability to infect *in vivo* (see section 2.2.2 Ch3) would make this less likely as discussed above. Physical damage to the shoot tips during preparation for interaction *in vitro* may be a cause of the lower frequencies of coiling. Shoot tips are handled with forceps and this may cause damage which reduces their ability to infect.

When shoot tips were inoculated *in vivo* they were securely fastened to *Pelargonium* petioles with strips of autoclave tape. Two of the *in vitro* inoculation methods involved shoot tips being fastened down or secured in position against host petioles and two did not (see section 2.4.3 Ch3). Unfastened shoot tips were more likely to move from the optimum position they were orientated in following inoculation than those which were securely fixed. When shoot tips were inoculated using the silicone rubber collar method (see section 2.4.3 Ch3) no coiling resulted. It may be that the collars exerted too much pressure on the fragile shoot tips and damaged them making coiling impossible. The glass capillary method (see section 2.4.3) involved pushing shoot tips down a narrow glass tube. Despite the care taken this may have caused abrasion which affected the

ability of the shoot tips to coil. The advantage of the glass capillary method was that the apex of the shoot tip could be precisely orientated and firmly held in position against the host. The simple alignment method (see section 2.4.3) reduced the amount of manipulation of the delicate shoot tip. However there was no firm fixing of the apex against the host. Similarly when cultured shoot tips were orientated against the host they were not firmly fixed into place. They also underwent much physical manipulation in the preparation for culture and inoculation.

The frequencies of coiling for three of the different *in vitro* inoculation methods were very similar. It may be that the frequencies for each method were determined by a combination of whether shoot tips were firmly fixed, how much physical manipulation was involved or some other unidentified factor or factors. However, it may be that a firm fixing was not important and the physical manipulations did not damage the shoot tips at all and it is other unknown factors which limited the frequency of infection *in vitro.* The effect of light quality, photoperiod, temperature and humidity on the frequency of coiling and infection have not been examinied. Some of these environmental factors may have some influence.

A more thorough examination of how the parasite shoot tip responds to the host may have enabled a more successfull inoculation procedure to be developed. A preliminary experiment was formed and is described in Appendix 4.1.2.4.

I.2.4 The perception of the host

The experiment which examined the sensitivty of different points of the shoot apex to tactile stimuli (see Appendix 4.I.2.4.) may be interpreted in various ways.

(I) Shoot tips are not sensitive to touch 0.5, 1.0, 2.0, 3.0 and 4.0 cm from the apex.

(2) When the parasite is stimulated around the complete circumference simultaneously the parasite does not respond. It may be that the shoot tip requires stimulation at one side only before it will respond by coiling.

Fritshe *et al.* (I958) found that the region most sensitive to a tactile stimulus was that nearest the apex. The experiment described in Appendix 4.1.2.4 may not have investigated close enough to the apex. The fact that the shoot tips used in the experiment did coil around each other when they subsequently came into contact with each other shows that they were able to respond when the stimulation was correctly applied. Perhaps the most suitable way of examining the response of the parasite to a host would be to record the events with a time-lapse video This would show exactly where and when the shoot tip begins to coil.

The problem of low frequencies of coiling and infection *in vitro* is partly overcome by inoculating several shoot tips onto a single petiole. This increases the chances of obtaining a successful infection. Further work may enable a more suitable inoculation procedure to

be used but the present system is adequate.

1.2.5. The influence of the *in vitro* procedure on the way the parasite invades the host

The similarity of the structural changes which occur during *in vivo* and *in vitro* interactions (see section 2.5. Ch3) demonstrates that the latter is representative of the former. The search hyphae of parasites infecting cultured hosts *in vitro* are seen to become lignified in the same way as those which infect *in vivo.* Tsivion (1978) found that the trigger for the development of this lignification is contact with the host vascular system. Fig. 2.4.2. in Ch3 showed that the *in vitro* shoot tips are able to reach the tip advance stage. This confirms that functional connections (nutrients can be withdrawn from the host plant) can be formed between host and parasite *in vitro. Cuscuta* plants act as very powerful sinks of host nutrients. Wolswinkel (1977) showed that an infecting parasite was able to prevent fruit formation in the host. In the *in vitro* system cultured petioles have limited endogenous resources as they have been severed from the rest of the plant and do not form roots into the growth medium. The growth of *Cuscuta in vitro* must therefore be limited unless the petiole is able to obtain sufficient nutrient from the growth medium. It was not known whether the petioles were able to gain any nutrients by photosynthesising because no measurements were taken.

1.3. The affect of culturing and the *In vitro* conditions on the host

I.3.1. The change in the chlorophyll (chl) concentration and content of petioles during culture

In several experiments (section 2.3.1 to 2.3.4 of Ch3) chl concentration and content per petiole was used as indicators of the physiological state of petioles *in vitro.* Changes in chl concentration have often been associated with the senescence of leaves (Nooden and Leopold (1978); Simon (1968); Thimann (1985) and Fleck *et al.* (1986). Another important aspect of the chl concentration in leaves is that it is related to the amount of incident light.

The difference in the chl content of freshly excised petioles at the same physiological stage (taken from emerging leaves with newly expanded leaves) but harvested at different time of year can probably be best explained in terms of the incident light. The plants from which petioles were excised were grown in the greenhouse. The petioles which received the most light would be expected to have the highest chl concentration. Even though supplementary lighting was provided in the winter plants would be expected to receive more light in the summer months. The higher chl concentration in petioles harvested in February (see section 2.3.1 to 2.3.4) may at first seem strange. However it should be remembered that in more vigorous plants the canopy of leaves is denser and

therefore petioles are more shaded. Hence petioles produced in the summer probably receive less light.

The variation in the chl concentration of freshly excised petioles made it necessary to take samples each time an experiment was performed. Unfortunately this was not done for the experiment in section 2.3.3. Therefore care should be taken in interpreting changes in the first few days.

A fall in the amount or concentration of chl between days 0 and 3 seen in the experiments in section 2.3.4 may not signify that the petioles are undergoing senescence. Instead it may signify that the chl is changing to match the lighting conditions inside the cabinets. In the experiment in section 2.3.2 Ch3 the rapid rise in chl concentration probably signifies a higher light intensity in the growth room than had been incident on the petioles in the greenhouse. The different pattern of change of chl concentration for cultured petioles in this section probably reflects the different growth room and culture procedure. Early decreases in the chl concentrations of cultured petioles found in some experiments may be avoided by increasing the light intensity inside the growth cabinets.

The lack of a significant change in the chl concentration after 3 days for petioles supplied with 0.2mg l^{-1} kinetin (see section 2.3.3 Ch3), while there was a significant fall for those which were not supplied with it suggests that kinetin delays changes in chl. The significant decrease in chl concentration for petioles cultured without kinetin does not necessarily mean chl is being lost. There are several possible reasons for the significant decreases in the chl concentration:-

(1) the amount of chl in the petioles is decreasing.

(2) the fresh weight of the petioles is increasing while the amount of chl is (a) decreasing; (b) staying the same; (c) increasing at a slower rate than the fresh weight.

The lack of a significant change in the chl content of petioles after 3 days (with the exception of petioles treated with 0.4 mg l^{-1} kinetin (see section 2.3.4 Ch3) shows that the actual amount of chl in petioles did not decrease with time in culture .

As will be discussed below much of the change in the dimension and fresh weight of petioles occurs at their ends. These ends are covered in translucent plastic caps containing agar. Chlorophyll is less likely to be produced under these shaded conditions. Indeed the new tissue is noticably white (see section 2.3.3 Ch3) . The measurements of petiole fresh weight and dimension, in combination with the results showing no significant change in the amount of chl, suggests that the decrease in chl concentration in petioles cultured with kinetin was due to an increase in the ratio of fresh weight to chl in cells or to the production of new non chlorophyllous tissue, rather than to actual chl loss.

The increase in the mean chla content per petiole with time in the experiment in section 2.3.I. shows that there was net synthesis of chla. This was not found in petioles under the

same conditions in the experiment described in section 2.3.3. The difference between these experiments may be seasonal. Even though the culture conditions and the physiological ages of the petioles were apparently identical they may respond seasonally to some endogenous factor.

The significant fall of the chla and b contents of petioles cultured with 0.4 mg l^{-1} kinetin after six days suggests that kinetin has accelerated chl loss compared to petioles cultured without it. This does not correspond with the literature which implicate kinetin as a retardant of senescence e.g. (Thimann 1985 or the experiment in section 2.3.3). The descrepancy between the two experiments may reflect seasonal differences in some endogenous factor which governs the response of the plant material to growth substances. The finding that the higher concentration of kinetin (0.4 mg l^{-1}) apparently speeded up chl loss is not consistent with the work of Thimann (1985) either. The 0.4 mg l^{-1} concentration of kinetin used corresponds to approximately 2μM. The concentration used by Thimann (1985) to retard chl loss was 15 μM. Further experiments with higher concentrations of kinetin must be done to determine whether the results of the experiment performed in this thesis were an anomaly or representative of the way *Pelargonium* responds to kinetin. The changes in fresh weight, dimension and protein concentration for petioles cultured in various ways are discussed below.

1.3.2. The change in the fresh weight and dimensions of cultured *Pelargonium* petioles.

Increases in mean fresh weight combined with changes in dimension suggest that the petioles (See sections 2.3.3 and 2.3.4 Ch3) were not senescing but actively growing during the ten days of the experiments. Petioles did not increase in length to any significant extent during the culture period. The end pieces, the only regions which increased in diameter during culture must therefore be the sites of weight accumulation.

The greater increases in diameter of the end portions of petioles containing kinetin and IAA (see section 2.3.3 and 2.3.4 Ch3) as opposed to with IAA only, suggests that both growth substances have a role in the expansion of the cut ends. Unfortunately measurements were not taken for petioles cultured without any growth substances therefore it is not possible to say whether expansion and increase in fresh weight occur when they are absent.

The mean diameter of the top region of petioles cultured with 0.4 mg or 0.2 mg l^{-1} kinetin were not significantly different from each other. This suggests that the higher concentration is no more stimulatory than the lower concentration.

Over the ten or eleven day culture periods the increases in the mean fresh weights were less than double what they had been when they were excised. The small increases were probably due to the expansion of the petioles being confined to an increase in the

diameter of the end portions.

Another symptom of the onset of senescence in leaves is a rapid decrease in the soluble protein (Simon 1968, Fleck *et al*. 1986 and Thimann 1985). Changes in the protein content in extracts of petioles which were cultured for different lengths of time are discussed below.

1.3.3. The protein content in extracts of freshly excised and cultured *Pelargonium* petioles

The lack of significant differences between the protein contents of extracts of freshly excised and three and five day cultured petioles (see section 2.6 Ch3) suggests that senescence is not taking place. A decrease in soluble protein is a primary event in the senescence of certain plant tissues (see Fleck *et al.* 1986).

The difference in the mean protein concentrations of the middle and end portions of cultured petioles may reflect the expansion which takes place at the ends. Protein may not be increasing at the same rate as fresh weight thus giving smaller values per unit fresh weight than the middle regions which are not expanding. Unfortunately the middle and end portions of freshly excised petioles were not analysed separately for protein content so it cannot be said whether petioles have a similar portein distribution at this stage. A shortcoming of the data is that they only describe protein concentrations up to 5 days in culture. Assays performed later may have shown subsequent decreases or increases in protein which would show whether senescence might be occurring. However it can be concluded that up to 5 days in culture there is no indication that petioles are undergoing senescence.

Taking all the parameters discussed here into consideration (changes in chl, fresh weight, dimension and protein) there is no evidence that petioles begin to senesce when they are excised from the rest of the plant and are placed in culture.

The middle portions in particular undergo little change. However the end portions of the petioles become much modified in response to their new environment. The transverse section of a cultured petiole described in section I.2 Ch3 demonstrates that the structure of the middle portions of cultured petioles retain their normal anatomy.

The experiment measuring the CE activity in cultured and uncultured petioles (see section 2.6 Ch3) was designed to determine whether the culture procedure caused any biochemical changes which might affect the invasion of the parasite. This experiment is now discussed.

1.3.4. The effect of the culture procedure on the endogenous cellulase activity in petioles of *Pelqrganium zonale*

As cell wall degrading enzymes were thought to be involved in the host/parasite

interaction (Thomson 1925, Nagar *et al.* 1984) it was important to know if the culture procedure itself changed their activity. An increased enzyme activity in the host might alter the capacity of the parasite to invade the host.

The low CE activity in *Pelargonium* petioles (see section 3.1.3 Ch3) made it difficult to measure. The increase in the magnitude of CE activity and the frequency of its occurence in the end portions of five-day cultured petioles relative to freshly excised petioles suggests some stimulation takes place in culture. This stimulation is consistent with work in other plant systems where an application of auxin has been found to stimulate the measured activity of CE and other cell wall degrading enzymes (Abeles and Leather (l971); Datko and Maclachlan (l968) and Ferrari (l972)). Datko and Maclachlan also found that the increase in activity coincided with enlargement of the region. The greater frequency and magnitude of CE activity in the end portions of cultured petioles shows that it is the expanding growing parts which experience the stimulation in CE activity. The activity in the middle portions of the 5 day cultured petioles was similar to that found in freshly excised petioles. The middle portions are the parts which are actually penetrated by invading haustoria. Therefore it may be that the parts of the petioles which are most important in the study of this host parasite interaction are unchanged with respect to CE activity. However it it must be emphasised that is not known what happens after five days in culture and the possibility that the region of stimulated activity may extend to the middle portions cannot be discounted. As it seems that auxin stimulates the activity of cellulase in cultured petioles it would be advisable to examine its influence on the activity of other key cell wall degrading enzymes which might alter the capacity of the parasite to invade, such as those which degrade pectin.

Having looked at the impact of the *in vitro* procedure on the host and parasite the usefulness of the system is now discussed.

1.4. The usefulness of the *in vitro* system that has been developed

Many of the potential advantages of a proposed *in vitro* system described in Ch1 have been realised during the development of the *in vitro* system.

1.4.1. The choice of plant material

Interacting single parasite shoot tips with single host petioles (see section 2.1 Ch3) means that attention is focussed on the important parts of the plant for a study of cellular interactions. The standardisation created by choosing *Pelargonium* petioles of the same physiological age from genetically identical plants and *Cuscuta* shoot tips derived vegetatively from the same parent plant are likely to reduce the sources of variability.

1.4.2. The *in vitro* environment

Enclosing each host/parasite interaction (see section 2.4. of Ch3) in a plant container gave much flexibility with respect to the manipulation of the external environment. Individual interactions could be easily moved to a different set of environmental conditions. However, the large volume provided by each container may not have been necessary. The availability of space in the growth cabinets limited the numbers of plant containers thus the number of interactions which could be placed in a given set of environmental conditions. Using containers with smaller widths would have been able to remedy this situation.

Each container provides an enclosed environment. Therefore it is possible to control the gaseous and chemical environment of individual interactions under the same lighting and temperature conditions. The potential for chemical control via the growth medium is discussed in section 1.2 of this chapter (4). The containers provide a safe, managable space of known volume within which to perform radioactive experiments.

The moisture content was shown to be a limiting factor in the infectivity of shorter (<60mm) *Cuscuta campestris* shoot tips. Some shoot tips dry out before they complete all the necessary stages and successfully infect. During petiole culture each plant container holds approximately 200 ml of agar medium in its base. This generates a high humidity inside the container which is likely to slow the drying out of any inoculum. The high humidity may be an advantage, increasing the probability of shoot tips reaching the later stages of infection once coiling has been achieved.

1.4.3. The suitability of the system for performing enzyme assays

The majority of the results of the enzyme assays described in section 3 of Ch3 are for *in vivo* interactions. However, assays of *in vitro* material should be no more difficult to perform as the procedures will be the same. Since the parts of the host and parasite plants not included in the assay have already been removed the time involved in harvesting is reduced.

1.4.4. Disadvantages of the system developed

There are two main disadvantages with this *in vitro* system. Firstly the time required to sterilise and set up the cultured petioles and parasite shoot tips is considerable. However, the investment in time is necessary to achieve the great benefits of this approach. One possible way of reducing the time involved would be to modify the way agar medium is applied to the top of the petiole (see Fig 1.4.4.). Medium could be supplied in larger quantities from a dish type structure containing an 'anti-slumping' mesh to prevent agar falling out. This would reduce the number of changes of medium required. The low frequency of coiling is another drawback of the system. This has been discussed

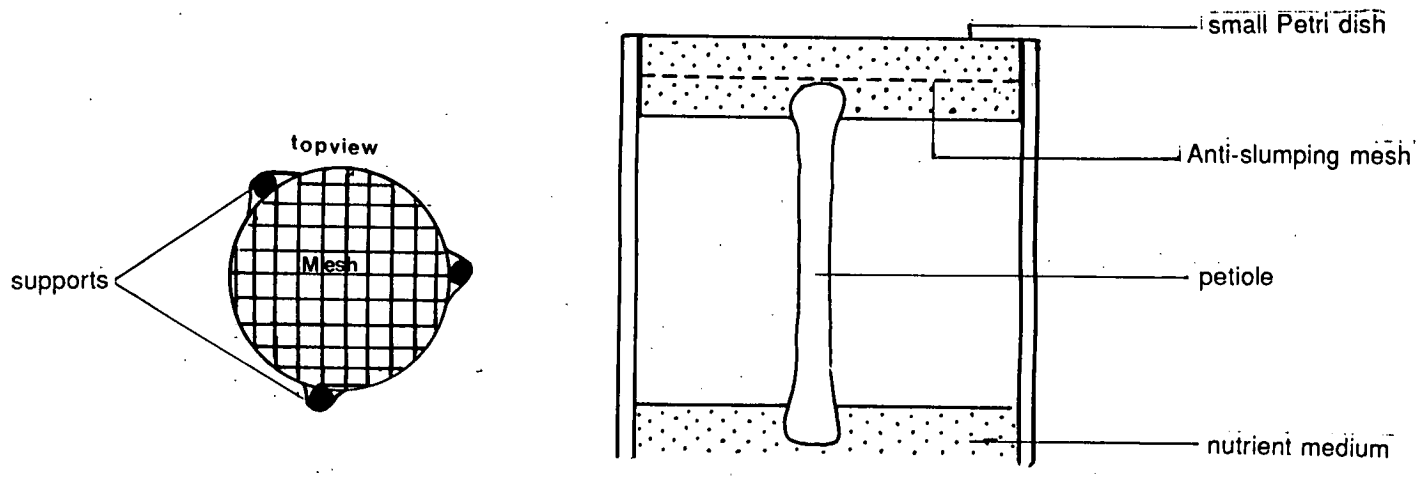


Fig 1.4.4 A diagram illustrating the proposed modification to the culture procedure for Pelargonium petioles.

in section 1-3 of this chapter. It was pointed out that this could be compensated for by increasing the number of inocula. However a technique for improving the frequency of coiling would be more desirable.

SECTION 2

A Discussion of the host/parasite interaction at the organismal, cellular and molecular level based on results obtained *in vivo* and *in vitro*

For the purpose of this discussion the interactions which take place between host and parasite have been split up into three sections, Interaction at the organismal, cellular and molecular levels. It is recognised that to some extent it is an artificial subdivision because each level of interaction is bound up with the other two. However viewing it this way may make it easier to describe and understand.

2.1. Interactions at the organismal level

2.1.1. The formation of tight coils

A feature of the coiled region formed by *Cuscuta* around its host is its tightness. This tightness presumably provides a strong anchored position from which to exert pressure during the penetration of the host. The significant increases in the coil length as shoot tips increased in length between 40 and 60 mm (see section 2.1.3 of ch3) suggests that there is some relationship between the two parameters. The length of the inoculum must in some way limit the coil length for shoot tips 60mm long or less. It is not known how the coil length increases for shoot tips longer than 60 mm. Presumably, the longer the coil length the more secure the anchorage and the longer the region available from which haustoria can arise. However, it is unlikely that the coil length will increase with the increase in the length of the inocula indefinately. The division of resources is presumably well co-ordinated so that too much energy is not expended on any one stage. It is important to coil and to form a rigid base for penetration but sufficient resources must remain for the subsequent penetration itself and the inoculum must survive several further days before vascular connections are made with the host. Truscott (1958) found that whole plants could be regenerated from individual haustoria following damage. Large numbers of haustoria may not be essential for a successful infection. However larger numbers may reduce the time taken for the parasite to complete its life cycle.

The restriction of coiling activity to the period immediately following inoculation, followed by a temporary dormancy of the apex (see section 1.1 Ch3) is presumably a way of using the limited resources in the most economical way. All the available nutrients can be channelled into penetrating the host. This pattern of invasion by *Cuscuta* shoots has also been described by both Kujt (1969) and Dean (1937) confirming that what was observed here is a general phenomenon.

2.1.2. The significance of the changes in colour and papillate appendage formation during invasion by *Cuscuta campestris*

The change in the colour of the parasite stem from orange/yellow to green and back again as shoot tips become established on *Pelargonium* petioles, may be due to several factors. The increase in the green colour may be due to:

(1) an increase in a green pigment

(2) a decrease in a masking yellow or orange pigment or

(3) both of the above

If the green pigment were chlorophyll (chl) it is possible that it was synthesised to permit photosynthesis and so supplement the energy reserves at this very critical time during penetration. The development of the green colour coincides with the time the parasite is penetrating but before connections have been made (See section 1.1 Ch3). Transverse sections of *Cuscuta* stems show air spaces which will allow gaseous diffusion inside the stem but no stomata. The papillate appendages which form on the tight coiled regions stomata like apertures and sub-stomatal air chambers beneath. these are probably specialised organs which allow the gaseous exchange necessary for photosynthesis. The appearance of these organs coincides with the development of the green colour and the time when penetration is occurring. All these factors together suggest that photosynthesis could be occurring at this point.

Greening of *Cuscuta* stems under stressful circumstances has been reported elsewhere. Zimmermann (1962) and Macleod (1976) found *Cuscuta* stems became very green under poor lighting or starvation conditions. For *Cuscuta campestris* the development of the green colour was found to be as a result of the formation of chl containing chloroplasts (Macleod 1961). Lyshede (1985) affirms that *Cuscuta* stems produce chl when under stress. *Cuscuta epithymum* attached to a dead plant of *Medicago sativa* taken from an infested field lived for several weeks but changed in colour from red to pale green (Lyshede 1985). In their review Ashton and Santana (1976) stated that no stomata are found outside the infecting stage. They are presumably referring to the stomata bearing papillate appendages which were found in this project to occur only on the tightly coiled regions. The greening of the coiled region during invasion is most likely to be as a result of nutrient stress as described by Macleod (1961). The lighting conditions remained the same throughout the experiment as the stems changed from orange/yellow to green and back again. It is therefore unlikely that the lighting was responsible for the colour change.

It would be interesting to establish whether photosynthesis was occurring and to compare its rate with that of non-parasitic autotrophic plants. It may be that the parasite was able to meet many of the increased needs during penetration by becoming temporarily autotrophic.

As greening coincided with times of stress for *Cuscuta campestris* shoot tips, so the

change back to orange/yellow coincided with events which indicated that the stress was over. The change in colour back to orange/yellow followed the tip-advance stage when the parasite is taking nutriment from the host and using it for growth. Thus the colour changes of *Cuscuta* stems would appear to be good indications of the physiological states of the parasite tissue.

2.1.3. The development of prehaustoria

The development of prehaustoria between the tight coiled parasite stem and the host petiole presumably maximises the close contact of the host and parasite cells. Their growth must also increase the pressure and increase the tightness of the grip the parasite has on its host. The repeated lobing of the superficial cells of the prehaustorium increases the surface area of the parasite. This increase in area may enable the parasite to completely mould itself to the surface of the host. It may also maximise the surface area available for secretion of the substance which produced such a smooth interface between the two organisms (see section 1.1 Ch3). The nature of this substance is unknown. Weinert and Barckhaus (1975) described a fortified deposition of cutin in spaces of the outermost layer of cell walls between *Cuscuta* and its host. It may be that the substance observed here was cutin. The layer may act as a sort of adhesive, sticking host and parasite together. Exudates at the surface of invading structures of higher plant parasites are not only found in *Cuscuta* . Olson and Kuijt (1986) found a mucilagenous exudate at the surface of invading organs of *Viscum minimum.*

2.2. Interaction at the cellular level

Prior to penetration host cells are not damaged or challenged. Cellular interactions proper start when the parasite begins to push its cells deep inside the epidermis of the host.

2.2.1. The initial penetration of the host epidermis

The neat, punch-like penetration of the host epidermis shown by SEM (see section I.2 Ch3) suggests the parasite enters host with a minimum of lateral disruption. The collapsing of host cell walls on top of each other in the front of the invading haustorium (see section 1.2 Ch3) must require physical pressure. It is not inconcievable that the parasite releases, or induces the action of, cell wall loosening enzymes which would make the process easier.

Thomson (1925) described the penetration process of *Cuscuta reflexa* in various hosts, suggesting it was performed by a combination of enzymic degradation and pressure. The observations made here have also been interpreted in this way. The involvement of specific groups of degradative enzymes in penetration is discussed in more detail in

section 2.3.

2.2.2. The penetration of the host by inter- and intra-cellular movement

As found in all other work on *Cuscuta* the main body of the haustorium was observed to stop growth after a short distance and superficial cells grow on as individual filaments. The majority of these filaments or search hyphae move towards the centre of the petiole and penetrate the sclerenchyma layer. This inwards growth towards the centre of the petiole may be for at least two reasons. Firstly the search hyphae may be preprogrammed genetically to grow in parallel with the length of the haustorium. Secondly they may be responding to some stimulus inside the host plant. The change in direction of growth at right angles to their original path towards the vascular bundles following penetration of the sclerenchyma layer (see Fig 1.2o and Fig 2.5c) suggests that the parasite is guided in some way. This is supported by the work of Lackey (1953) who claimed the positioning of haustoria and their growth was governed by the position of vascular bundles in the host.

Intercellular movement. The movement of *Cuscuta* search hyphae between cells was particularly apparent when they traversed the sclerenchyma layer see Fig 1.2 Ch3. The complete ring of sclerenchyma surrounding the vascular tissues did not in the majority of cases appear to be a significant barrier to the advancing parasite. In Fig 1.2o of section 1.2 of Ch3 the search hyphae of one haustorium appeared swollen and compressed against the sclerenchyma while those of the adjacent haustorium had penetrated it. This apparent difficulty in crossing sclerenchyma layers has been observed by others. Kuijt (1969) reported that sclerenchyma layers have been found to render *Digitalis* and *Quercus* plants resistant to attack by *Cuscuta*. Complete resistance is clearly not conferred on *Pelargonium* by possession of such a layer. It may be that in these other species the layer is much thicker or has greater mechanical strength.

The TEM micrographs show that the parasite is able to deform itself sufficiently to move into the small gaps at the junction between two host sclerenchyma cells. The parasite looks like a wedge attempting to prise the two cells apart. It may be that pressure plays a major part in the crossing of sclerenchyma layers. This would explain why the parasite might fail to cross thicker layers. The parasite may not possess the necessary enzymes to separate two sclerenchyma cells relying on pressure alone.

Once inside the central parenchymatous region the parasite appears to travel both inter- and intracellularly. The combination of the two routes suggests that the parasite has no preference for one or the other. TEM observations made by Dorr (1968) are in agreement with those made here.

Movement of the parasite between cells is likely to involve both enzymic degradation and pressure pushing cells apart. In contrast to the initial penetration, cells either side of the

parasite do not appear to be collapsed or destroyed. Little generalised disruption of the host is apparent and this approach by the parasite would seem less likely to invoke any wounding or resistance response in the host.

Intracellular movement. The light micrographs in section 1.2. Fig 1.2o,p and r strongly suggest that *Cuscuta campestris* hyphae penetrate host parenchyma cells. The light micrographs in section 2.5 also show this. The TEM micrographs in section 2.5 confirm that *Cuscuta* hyphae enter host cells. Host cells walls are not apparently perforated by the hyphae but become invaginated in front the advancing parasite. A host cell wall is visible at all points in front of the invading hyphae. Invagination of the host cell may be expected to be caused by the following:-

(1) The parasite may produce cell wall degrading enzymes which soften the host wall and make it more elastic.

(2) The parasite may induce host cells to produce its own cell wall loosening enzymes which allow it to become more elastic. In order to maintain a covering over the advancing parasite tip the host must synthesise cell wall.

(3) Neither of these.

The lobing of the parasite into the host cell seen by TEM (see Fig 2.5.h) suggests that pressure is being exerted. Again a combination of physical pressure and enzyme degradation are implicated in the invasion of the host.

The appearance of the parasite moving into the host cell is reminiscent of penetration by fungal hyphae. The thickened host cell wall at the point of entry into the host cell is a common feature in fungal penetration (Bracker and Littlefield 1973). However the similarity stops here. *Cuscuta* does not appear to make a cut in the host wall and enter the cell as fungi are shown to do. Absent also from *Cuscuta* parasitism is the extracellular matrix which separates the invading cell walls from the membrane of the host cell (Bushnell 1972). Technically, *Cuscuta* does not actually appear to enter the host cell, staying outside the wall. Dorr (1968) claimed that the cell wall of the host did not remain intact. If the host cells walls are broken and the parasite wall comes into contact with host plasmalemma it must be a very short lived stage because even at the extreme edge of the parasite invading the cell in Fig. 2.5.h of Ch3 a distinct host wall is evident.

Outside the host cell the close proximity of cell walls of host and parasite shown in Fig. 2.5.h and *g* of Ch3 demonstrate that there is no intermediate layer between the host and parasite. Even at the higher magnification of Fig. 2.5.h there is no visible intervening layer which is either more or less electron dense than the two opposing cell walls. The different staining properties of the two walls showed that they differed in some respects from each other. A matrix often develops between host and parasite when intercellular fungal parasites enter a tissue (Held 1972, Olah *et al.* 1971) and the host develops wall

appositions (Heath 1972). These are not seen here.

In higher plants when unwounded cells come into contact they respond to each other in a number of ways. Calli of different plant species will react to each other by forming a nectrotic barrier, closely adhering to each other or not responding at all (Fuji and Nito 1972). Some grafting partners although totally unrelated will form plasmodesmatal connections. (Kollman and Glockmann 1985). These may however be short lived and the interaction can be followed by expression of symptoms of incompatibility.

Different species of higher plant may tolerate contact with each other more readily than they will contact with fungi. Compatibility between foreign cells will be discussed in more detail in section 2.4.

The apparent mix of cytoplasm and vacuole contents in Fig 2.5 of Ch3 may be as a result of several factors. One possibility is that the host cell is breaking down as a result of the entry of the parasite. Such membrane breakdown is commonly found in host/parasite interactions involving fungi (Ehrlich and Ehrlich 1971) bacteria (Goodman 1972) and viruses (Esau 1968). Another possibility is that the membranes were disrupted during the fixation of the material while being prepared for microscopy. A third possibility is that the tissue culture procedure disrupted the cell. The latter two possibilities could be checked by viewing more cells in micrographs of uninfected *in vivo* and *in vitro* petioles.

Some of the molecular changes which occur in the host and parasite during invasion are now discussed.

2.3 Molecular changes during interaction between host and parasite

Before the results obtained are discussed it is appropriate to discuss some of the methods used and the way results were presented.

2.3.1. Discussion of methods and presentation

The freeze/thaw extraction procedure. The advantage of this procedure was that it was possible to assay very small amounts of plant material. Addition of sand and manual maceration of small samples (<lg) was found to lead to the loss of a major part of the extract. Without maceration the amount of protein yielded might have been expected to decrease. The results from an experiment comparing the two methods show this to be true (see Appendix 4.2.3.1) . It may be that certain types of protein are preferentially released or retained during the freeze/thaw procedure. The enzymes in the walls of cells are most likely to be released. These were the proteins of most interest in this thesis. As a consequence using the freeze/thaw method is unlikely to have compromised the work to any significant extent.

The use of "per coil" as a unit of measurment. Experiments in section 2.1.3 of

Ch3 showed that the length of coil formed around the host is to some extent dependent on the length of the inoculum. For a fixed length of inoculum the length of the coiling region was relatively constant. However there were frequent exceptions to the rule with extremely small coil lengths, very different to the rest. This should be taken into consideration when interpreting the significance of changes in quantities of a substance or activities of an enzyme on a per coil basis. The results should be taken as a general indication only.

2.3.2. The significance of the change in the protein concentrations in extracts of tight coiled regions of *Cuscuta campestris* between 2 and 11 days after inoculation

The significant fall in the protein concentration in tight coils of *Cuscuta campestris* between days 2 and 4 following inoculation (see section 3.1.2 Ch3) could be for at least two reasons:

(1) There was a net loss of protein.

(2) The relative proprotions of other cell constituents were increasing while the quantity of protein was either staying the same or increasing at a slower rate. The decreasing number of coiled regions per 80mg sample showed that the fresh weight of these regions was increasing with time after inoculation (see section 3.1.2. Ch3). It is likely that the fall in the protein concentration was related to this. The second explanation for the decrease in the mean protein concentration is therefore more likely.

The lack of any further significant change in the protein concentration after day 3 showed that the rate of change of protein from then onwards kept pace with the fresh weight. The continued fall in the number of coiled regions per unit sample suggest that there was some slight rise in fresh weight. There was no significant increase in the protein content per coiled region between days 4 and 10 (see section 3.1.2. Ch3) The protein remains relatively constant as the parasite becomes established on the host. The significant increase in the protein concentration between days 10 and 11 post inoculation occurs when *Cuscuta campestris* shoot tips have usually completed all the necessary stages of infection on *Pelargonium* petioles. Tip advance which signifies a withdrawing of nutrients begins on day 6 (see section 1.1 Ch3) The large increase in protein in the coiled region is most likely to be due to the parasite having made a functional connection with the host. The parasite will then be able to withdraw the building blocks of protein (amino acids) or the necessary resources to enable it to synthesise them. Macleod (1963) showed that *Cuscuta* was able to synthesise its own amino acids. However it did require at least a source of inorganic nitrogen from its host. The rapid increase in protein is presumably because the parasite has now obtained this necessary nitrogen.

2.3.3.The significance of the difference in protein in coiled and uncoiled *Cuscuta* stems

The difference in the amount of protein extracted from mature attached coiled regions and the uncoiled non-apical *Cuscuta* stem (see section 3.1.1 Ch3) are perhaps not surprising. The non-apical parts of the uncoiled stem may serve as channels between the advancing apex and the last coiled region and also to the same extent storage cells for accumulated nutrient. This region of the parasite might not be expected to have a very high metabolic activity and protein content. In experiments measuring DNA and RNA concentrations in the different parts of *Cuscuta* vine (Charles *et al.* 1978) this non apical non coiling region was found to have the lowest amounts. This would confirm that this region is the least metabolically active. In contrast to this, coiled regions which are the tissues from which the invasion of the host is mounted might be expected to have a much higher metabolic activity and protein content.

2.3.4. The protein content in infected and uninfected *Pelargonium* petioles and stems of *Cuscuta*

The similarity in the protein extracts of infected and uninfected petioles (see section 3.1.1 Ch3) showed that invasion by the parasite did not stimulate a generalised increase or decrease in the protein content of the host. The concentration of protein in any part of *Cuscuta* was greater than that for *Pelargonium* petioles. Petiole cells are in the main parenchymatous with large vacuoles. This is confirmed by the EM micrograph in Fig 2.5g in Ch3.

It would be interesting to know the protein concentrations of other parts of the *Pelargonium* plant. It may be that the petioles have the lowest concentrations. The larger protein concentrations in the least metabolically active parts of *Cuscuta* may be because the parasite stores protein in the same way that the plant obviously stores carbohydrate (see the large starch grains in Fig. 1.2a Ch3.

2.3.5. The significance of the change in cellulase (CE) activity in coiled and non-coiled *Cuscuta campestris* stem

The enzyme activity in extracts of *Cuscuta campestris* were expressed in three ways: per ml of extract, per mg protein and per coiled region. The activity per unit protein shows just how the actual activity of the enzyme changes, regardless of any changes in fresh weight. The activity per ml of extract takes into consideration the surrounding tissues of the plant and is possibly a better guide to the actual physiological activity of the enzymes. Activity per coil shows whether the activity of the enzyme changes with time for the whole organ but does not take into consideration any changes in fresh weight, nor does it show whether the increase was due to an increase in the quantity of protein or at

the level of activation.

The higher CE activity per ml of extract in the coiled regions of *Cuscuta campestris* (see section 3.1.1 Ch3) may be for at least two reasons.

(1) The increased activity may be connected with the maturation of the prehaustorium and /or the haustorium proper.

(2) The increase in CE may be because the enzyme is being used to penetrate the host.

Cellulases which loosen or break down cellulose have been found at elevated levels in many different circumstances. They have been implicated in ripening on certain fruits (Hatfield and Nevins (1986), Pressey (1977) and Hobson (1968), leaf abscission (Sexton *et al.* 1981) and parasite invasion (Bateman (1969), Kelman and Cowling (1965). Nagar *et al.* (1984) found a high CE activity in the haustorial bearing coiled regions relative to the non coiling region of *Cuscuta relfexa* parasitising *Lanata camera.* Cellulase enzymes have been found not only to act on cellulose but also on xyloglucan a hemicellulose (MacNeil *et al.* 1984). Cleavage of this wall component is thought to be essential during cell elongation (MacNeil *et al* 1984). Cellulases produced by the parasite may render host cells more elastic by cleaving hemicelluloses in their walls. The measurement of CE at different stages of the host parasite interaction (see section 3.I.2 Ch.3) and being able to relate these results to the observed morphological structural changes (see sections I.I and I.2 Ch.3) is a very useful extension of the work of Nagar *et al.* (1984). The large increase in the CE activity per ml of extract, per mg protein and per coil two days after inoculation and then a decrease again reaching a minimum at about eight days (see section 3.1.2. Ch3) correlates with important events in the parasitism of *Cuscuta* (see section I.I and I.2 Ch.3). The maximum activity reached after 4 days coincides with the time when the parasite is actually penetrating the host. By this time the prehaustorium is well developed and it is the haustorium which is growing in size during active penetration. The increased activity per ml of extract between day 2 and 4 amounts to approximately 1200%. Cellulase enzymes are usually found in the walls of plant cells (MacNeil *et al* . 1984). This large increase in activity in the walls of the coiled region and the haustoria might be expected to have some impact upon the walls of the adjacent host cells. Unfortunately the experiment under discussion only measured the change in the CE activity of the whole coiled region. If CE is to be implicated in the penetration process as a wall loosening agent it must be shown to be present at the interface between the two organisms. Simple histochemical procedures designed to show CE activity in plant tissues are not considered reliable enough. The published methods (Bal 1974) rely on staining of reaction products which are soluble. They are therefore likely to move around in the tissue before observation can be made and give misleading results. A preferred method is an antibody technique similar to that developed for locating CE in abscission zones (Sexton *et al.* 1981). This is initially very time consuming but is a very

powerful tool for accurately localising an enzyme in tissues. Until this work is done it cannot be conclusively proved that the rise in CE activity is not due in some part to the differentiation of the haustorium. The large standard errors in the mean CE activities per ml of extract, per mg protein and per coiled region on days 5, 6 and 7 (see Fig. 3.I.2. d, e and f Ch.3) are probably due to individual coils in samples not being in synchrony with each other. Each coil probably experiences a rapid increase then a rapid decrease in CE activity. However, the rapid fall in activity in some coils may coincide with the maximum or near maximum activity in other coils in other samples. Another possibility could be that certain coils have elevated CE activity for longer periods than others. This also would lead to these large standard errors. It is clear that there was a rise in activity between days 2 and 3 and between days 3 and 4. However whether there really was a fall in activity between days 5 and 6 and then a rise between days 6 and 7 is doubtful. The cellulase activities on days 6 and 7 per mg protein and per coil were not significantly different from each other. If the experiment is performed *in vitro* the precisely controlled environment and standardisation may allow the shoot tips to move through infection in a more synchronous manner. The changes in the CE activity in *Pelargonium* petioles are now discussed.

2.3.6. The cellulase (CE) activity in infected and unifected petioles

The extremely low levels of CE activity in extracts of uninfected petioles are what might be expected in a plant tissues which are not growing or changing in any way. The lack of any generalised increase in activity on invasion by the parasite suggests that the process did not significantly stimulate the enzymes activity. However, with this assay of the whole tissue it is impossible to demonstrate any localised stimulation of the enzyme which may have occurred. Such localised changes may only be effectively shown by some immuno histochemical method described in the previous section.

The results of Nagar *et al*. (1984) do not agree with those found here. They found that the CE activity in the tissues of the host surrounding the points of invasion by *Cuscuta reflexa* was greater by factors of 2-8 times than that for similar regions of infected plants. If there was an increase of this magnitude in *Pelargonium* petioles it might be expected to result in an increased frequency of detection (as found in petioles cultured for 5 days in section 2.6 Ch3) However this was not found. Discussion now turns to another cell wall degrading enzyme.

2.3.7. The significance of the change in pectin esterase (PE) activity in the tight coiled regions of the parasite during infection

The decrease in the mean activity per ml of extract between day 1 and 2 after inoculation and then the increase between days 2 and 3 (see section 3.2.3) implies at least

two things. Firstly the amount of enzyme which is active decreases then increases again. Secondly that there was an increase in fresh weight which was proportionally greater than any change in the amount of active enzyme causing the overall activity to decline. As the number of coils per 40 mg sample decreased between days 1 and 2 (implying an increase in the fresh weight) the latter is more likely. The fall in PE activity per ml of extract between 2 and 3 days after inoculation described in section 3.2.4 of Ch3 probably occurs for the same reasons.

The change in the amount of activity per mg protein was the same for coils of the same age in different experiments while the change per ml of extract was not. The change in fresh weight of the coiled region may be influenced by the environmental conditions such as availability of water and the temperature. This may account for the discrepancies between the activity per ml of extract in extracts of coiled regions of the same age from sections 3.2.3 and 3.2.4. of Ch3.

The increase in the PE activity per mg protein with time up to day 7 (see section 3.2.4 Ch3) shows that the change in this enzyme's activity is not merely as a result of changes in the amount of protein present and its relation to the fresh weight. There appears to be a peak in activity at day 7, however it is not known what happens after day 8. Further experiments should be extended to cover the period beyond 8 days.

Nagar *et al.* (1984) found the PE activity was highest in the tightly coiled regions. This implied that there was some stimulation of the enzyme in the tissues involved in mounting the invasion of the host. This was not confirmed here.

The increase in PE activity per mg protein and per ml of extract with time up to day 7 may mean that the enzyme has some role in the penetration process. However the fall in the activity per ml of extract between days 2 and 3 may also have some significance.

In the interpretation of the changes in PE activity it is important to notice that the difference in the minimum and maximum values for PE activity per ml of extract represent a change of only 50% in four days. This is quite small in comparison to the 1200% change in CE activity per ml of extract over a two day period. It would be useful to know just how elevated is the activity in coiled regions compared to uncoiled regions. It is possible that the largest change in PE activity takes place in the first 24hr after inoculation. The different patterns of change of PE and CE activity suggest that their role in the penetration process is different.

Pectin esterase is known to have a role in cell wall degradation by certain plant parasites. It has been found in culture filtrates of pathogenic fungi and bacteria (Miller and MacMillan 197l). The enzyme is however common in many parts of healthy uninfected plants. It has been detected in fruits, leaves, stems and roots (Rexova-Benkova and Markovic 1976). The enzyme is usually found in combination with polygalacturonase (PGURASE). Pectin esterase removes the ester group of esterified galactopyrosiduronic

residues of pectic polymers in plant cell walls. This makes the chain more amenable for PGURASE which only attacks non esterified residues. The action of the enzyme may also lower the pH in the cell wall as carboxyl groups are generated during the reaction. This would also make conditions more amenable for PGURASE which usually has an optimum at a mildy acidic pH.

If the enzyme is to be implicated in the invasion process it must like CE be precisely located at the host parasite interface. It is an enzyme which is situated in cellwalls. Any increase in its activity might be expected to have some impact on the walls of adjacent host cells. The best way of locating the enzyme would be an immuno histochemical method described in section 2.3.2 of this chapter. One confusing aspect and difficulty in interpreting the role of PE in the parasite is the apparent absence of PGURASE activity. This enzyme usually works in parallel with PE. This is discussed in the following section.

2.3.8 The possible reasons for, and significance of, the apparent absence of polygalacturonase (PGURASE) activity in extracts of the stems of *Cuscuta*

There are at least four possible explanations for the apparent absence of PGURASE activity in the coiled regions of the parasite:-

(1) The enzyme is not present.

(2) The enzyme is at a concentration in the tissues too low to detect

(3) The enzyme has a peculiar pH optimum.

(4) The enzyme was deactivated during extraction.

The complete absence of PGURASE is improbable as the parasite is likely to use this enzyme during its own development and growth. The success in measuring PGURASE in *Pelargonium* plants (see section 3.3.3 Ch3) and tomato fruits (Appendix 4.2.3.7) using the same method as was being used in this assay of *Cuscuta* tissues made it unlikely that the assay procedure was at fault. It is more likely that inability to measure PGURASE activity in extracts of *Cuscuta* was due to it being present in very low concentrations, as found for *Pelargonium* petioles (see section 3.3.3. Ch.3). If the enzyme is present at low concentrations the experiment performed for *Pelargonium* should be repeated for *Cuscuta*. The results in this thesis are not consistent with the work of Nagar *et al.* (1984). They found that PGURASE like PE and CE was detectable in all parts of the plant and its activity was elevated in the tight coiled regions. The pH optima of PGURASES are generally in the region pH 4.0 - 6.5 (Rexova-Benkova and Markovic 1976). Exceptions have been found with optima of 2.5 (Kaji and Okada (1969)) but they were in culture filtrates of fungi. Nagar *et al.* (1984) used a pH 5.0 buffer which is the same as one of those used in this work. The pHs of the buffers used in the experiments are therefore

likely to have been suitable.

Extraction procedures. The extraction procedures used in this study did not deactivate PGURASE extracted from *Pelargonium* or tomato fruits. The *Cuscuta* extracts used in the PGURASE viscometric assays were found to contain other active cell wall degrading enzymes. For example the solidification of reaction mixtures from which salts had not been removed revealed the presence of PE in these extracts (see section 3.2.1. of Ch3).

It is important to establish why PGURASE was apparently absent from extracts of *Cuscuta* tissue. If it was truly absent this would be very unusual and has extremely interesting implications. The use of PGURASE enzymes in the degradation and invasion of plant cell walls by pathogens has been shown to result in the production of biologically active molecules which activate a resistance response (see Davis *et al.* 1986). *Cuscuta* may avoid the use of such an enzyme during penetration as a way of avoiding triggering host resistance. Further experiments should use concentrated extracts, perhaps using buffers at other pHs' and the experiments should be run for longer periods. This may show whether the enzyme is at a very low level or is not being detected in the correct way.

The following section looks at *Cuscuta* parasitism in the wider context. Observations made in this thesis are discussed in relation to the invasion strategy employed by the parasite paying particular attention to the way the parasite invades its host eliciting only the minimum of response. The discussion is then continued with a section detailing future work which could extend the work performed here and explore some of the hypotheses made in section 3.

Section 3. The success of *Cuscuta* as a parasite

3.1. Constraints on *Cuscuta*

Although *Cuscuta* is a genus of highly successful parasites with complex adaptations to this habit there are certain things which restrict its success:-

(1) The environment limits the distribution of the parasite. It is not found in cooler temperate and cold climates (Kuijt 1969).

(2) The parasite can only infect stems or petioles.

(3) Generally it is confined to non woody stems finding it difficult to penetrate lignified tissues.

(4) It is known to be unsuccessful on hosts with highly acidic sap (Kuijt 1969).

(5) Infection can fail if the host plant actively resists invasion (see Tsivion 1979).

Herbaceous plants are abundant in most parts of the world and large areas of the world surface have suitable climates for the various *Cuscuta* species. The number of plants which have toxic sap must also be limited. However the problem of host resistance is possibly the most serious and important potential limiting factor. A successful parasite must be able to evade or combat the physical and/or chemical defence mechanisms of its host. Different parasites adopt different strategies. The more intimate the host/parasite interaction the more complex these strategies may have to become. Certain pathogens do not enter host plants to any significant extent. They rely on the diffusion of toxins and enzymes which kill and dissolve host cell releasing valuable nutrients. In contrast, *Cuscuta* like many other higher plant and fungal parasites penetrates deep into the living host tissues. In doing so *Cuscuta* must confront and attempt to evade or combat various levels of defence erected by the host. The response of the *Pelargonium* plant to invasion by *Cuscuta campestris* is now discussed.

3.2. The response of *Pelargonium* petioles to penetration by *Cuscuta campestris*

It was found in this project that *Cuscuta* stems form tight coils around the host and then penetrate it with absorptive feeding organs called haustoria (see section 1.2 Ch3). Host cells are apparently compressed on top of one another in the wake of the initial penetration. Later stages of intracell and intercell movement appear to involve a combination of pressure and enzyme degradation. Some, if not all, of these actions might be expected to generate some sort of response from the host. The only immediately obvious response was the swelling directly underneath the coiled regions of the parasite. The work of Dean (1937) confirms that these swellings are a common feature during the infection of plant by *Cuscuta* in the field. Olson and Kuijt (1986) write that proliferation of host cells in the region penetrated by higher plant parasites is a common occurrence.

The swelling of the petioles may or may not represent active resistance to the parasite. It may be a consequence of increased concentrations of growth substances caused by the presence of the parasite. One conceivable advantage to the host plant of an increase in girth is that the tight coiled region of the parasite could become ruptured. However Truscott (1958) showed that small numbers of individual haustoria have the ability to regenerate into a large parasite plant following physical damage or removal of the coiled regions. Unfortunately resin-embedded plant material taken from the swollen petioles was not sectioned and observed. Thomson (1925) found that *Pelargonium zonale* stems swelled when penetrated with *Cuscuta reflexa* haustoria. He also found that a lignified barrier was laid down, presumably in an attempt to exclude the parasite. This was not confirmed for the swollen petioles in this work.

The cell division in the outer layers of petioles infected with *Cuscuta campestris* (see section 2.5.1. Ch3) may have been due to the invasion by the parasite, or simply as a consequence of the culture procedure. Cell division stimulated by medium might have been expected to be somewhat more disorganised then the laying down of parallell cell walls seen in the outer parenchymatous layer of the host. Observations of uninfected, cultured petioles would be able to resolve this issue.

Perhaps the most interesting aspect of the swelling of the host is that its occurrence was not predictable in any way. There are several possible reasons for this:

(1) Variation in the way the parasite invaded the host. Under certain circumstances,

(a) the parasite produces something which stimulates swelling

(b) the parasite fails to produce something which normally suppresses swelling

(2) The incidence of swelling may also reflect the variation in the ability of the host to respond to the parasite.

In the experiment in section 2.1.2 of Ch3 several shoot tips were inoculated on to the same plant. In the several plants used it was not noted whether host swelling was confined to particular plants or whether it occurred in some of the petioles on a plant and not on others. This information may have helped to identify the likely cause of the swelling. If swelling was controlled by the physiological state of the host plant it might be expected that all or none of the infected petioles on a plant with multiple infections would swell at any one time. However, if the swelling of petioles was dependent on the physiological state of individual petioles or whether the parasite produced or failed to produce some chemical factor then it may be that only some of the petioles on a plant bearing multiple infections would swell.

The evidence in this work is that the *Pelargonium* plant was unable to actively resist invasion by *Cuscuta campestris.* The swellings of the petioles although an obvious reaction to the parasite did not significantly impede infection. No intervening layer detectable with light microscopy was found between the parasite and host cell walls during inter- and

intra-cellular movements.

There are examples in the literature of active resistance to *Cuscuta campestris.* The development of a lignified barrier around the parasite described by Thomson (1925) suggested resistance was taking place. Tsivion (1979) reported that tomatoes and certain bean varieties actively resist invasion by *Cuscuta campestris.* The reaction of tomatoes, which was particularly vigorous involving a hypersensitivity response, by no means always occurs. In California *Cuscuta* is a major pest of this crop plant (Ashton and Santana 1976). It is possible that the prolonged interaction of these two organisms has led to certain varieties of tomato evolving a resistance to the parasite. The various resistance responses to *Cuscuta* should be explored more thoroughly. An understanding of the mechanisms of this resistance might be useful in developing new methods for its control in areas where it is a serious pest.

The very wide host range of *Cuscuta* species (Gaertner 1950) and the fact that only sporadic reports of resistance arise in some hosts show what accomplished parasites they are. The following section looks at the way *Cuscuta* invades the host and attempts to identify factors which make it so successful. It is suggested that an important factor is the way in which *Cuscuta* manipulates cell/cell recognition between its own and the host cells.

3.3 The relationship between the mechanism of *Cuscuta* invasion and its success on a wide variety of host plants.

The apparent non-specificity of the coiling stimulus and haustorial formation must be one factor which enables *Cuscuta* to invade a large number of different host species. Any non-porous vertical cylindrical object appears to be sufficient to induce these responses. This characteristic might also count against it, because many man made objects are of the required shape. Clearly this was not a problem when the mechanism was evolved. In the natural environment man made objects are not usually a problem.

The intercellular and intracellular movements during the later stages of *Cuscuta* invasion appear to occur with the minimum of damage to the host. It would be tempting to suggest *Cuscuta* is successful on so many hosts because of physical stealth. However such thoughts are dispelled when it is remembered that the initial penetration of the host involved the mechanical crushing of many host cells. If this wounding induces a response (and perhaps the swelling which sometimes occurs is a manifestation of such a response) it is clear that this response does not amount to any successful resistance.

Alternatively it could be argued that the apparent compatibility of *Cuscuta* with many hosts is because higher plant cells have much more of an affinity for each other than they do for either fungi or bacteria. The fact that *Cuscuta* is sometimes resisted by its host (Tsivion 1979) would not support this hypothesis. Nor is this simplistic explanation

consistent with the evidence from grafting (Yeoman 1983) and pollen/stigma interactions (Heslop-Harrison 1975) that higher plants have the ability to differentiate between self and non self.

It seems likely therefore that *Cuscuta* has a mechanism which in the majority of circumstances prevents the host from undergoing a wound or resistance response. This mechanism may involve some form of cell/cell recognition or the blocking of normal cell recognition processes.

Recently it has been found that despite external appearances *Cuscuta* is unable to parasitise itself (Jacob *et al.* 1986). Growth of search hyphae is arrested in the cortex of the parasite which acts as a host, penetrated cells become deeply stained ('Osmiophilic') and degenerate. This suggests that *Cuscuta* is able to perceive that it is parasitising itself and to react appropriately. This is in marked contrast to the majority of its usual hosts. *Cuscuta* could therefore have some self -incompatibility system.

The sporadic swelling found in *Pelargonium* petioles as a result of attack by *Cuscuta* may result from some form of cell/cell recognition between host and parasite. Mechanisms of resistance in plants and how they may be triggered are now discussed. Ways in which *Cuscuta* may be able to overcome or avoid triggering resistance are also suggested from the results collected in this thesis.

Mechanisms and triggers of resistance to invading parasites

There is a vast amount of literature on the various responses of plants to attack by invaders. There is still little understanding of the means by which certain pathogens are able to live for prolonged periods inside host tissues while others are not. Each plant possesses physiological and structural characteristics which enable it to remain uninfected. These on the whole are present all the time. Parasites are able to avoid or overcome these resistances. The possession of certain wall degrading enzymes enables a parasite to traverse the structural (e.g. Bashan *et al.* 1985) barriers and acquired tolerance of chemical defences can enable parasites to successully invade host plants. Host plants do not only have defence mechanisms which are present all the time but also ones which can be triggered as a result of invasion. Plants may react to invasion with a hypersensitive reaction (Tsivion 1979, Kiraly and Bonna 1986, Maclean *et al.*. 1974), by forming lignin barriers (Vance *et al* .1980) or by modifying their wall with appositions (Aist 1977), or by a variety of other physical changes. As well as physical defences plants are known to produce anti-bacterial substances. A group of these substances are called phytoalexins (Dixon 1986). Recently a great deal of attention has been focussed on the activity of fragments of plant cell walls (usually short length oligosaccharides) in stimulating these defensive responses. Ryan *et al.* (1981 and 1986) found that small concentrations of fragments of plant and fungal cell walls produced by enzymic degradation activate genes which control the synthesis of plant defence chemicals such as antibiotic

phytoalexins. Extremely small concentrations of cell wall fragments have also been found to stimulate the production of anti-pathogenic substances (Darvill *et al.* 1984) and hypersensitive reactions (Moddermann *et al.* 1985). Fragments have been found to influence the morphogenesis of disorganised callus cultures of tobacco (Tran Thanh Van *et al* . 1985) and to inhibit 2, 4 D induced growth (Darvill *et al.* 1985). The have also been implicated as regulatory substances during flowering in *Lemna* (Darvill *et al.* 1985). The majority of cell wall fragments found to have biological activity are pectic in origin. However fragments of xyloglucan have also been found to be biologically active. Cellulase can degrade this ploymer (MacNeil *et al.* 1984). The high CE activity in the coils of the parasite during penetration may mean that the enzyme can potentially form active xyloglucan fragments.

Cell wall fragments and *Cuscuta* parasitism.

Observations and measurements made in this thesis strongly suggest that penetration of the host by *Cuscuta* involves enzymic degradation of parts of the host cell walls. Such action is likely to generate fragments of host and possibly some parasite cell walls. These cell wall fragments may be biologically active molecules potentially able to trigger wound or resistance responses in the host. It is suggested that *Cuscuta* could avoid such active wall fragments from invoking these responses in the following ways:-

(1) Generating wall fragments which are not biologically active

(a) no PGURASE was found in the coiled regions of *Cuscuta.* If this is truly representative of the real situation the *Cuscuta* could avoid the generation of pectic fragments, (the class of molecules which has been shown to be active in many different regulatory roles), by not using PGURASE to penetrate the host. However further experiments are necessary before this remarkable explanation is made.

(b) The parasite may be able to stimulate host cells to soften its own walls, in the process producing wall fragments which are not read as being symptomatic of injury or invasion and which induce no response.

(2) The parasite may avoid detection by the host by blocking receptor sites which could be stimulated by contact with wall fragments. Alternatively any cell wall fragments produced may be mopped up by the parasite preventing their detection and the subsequent response by the host.

This whole area of the role of cell wall fragments in cell/cell recognition between *Cuscuta* and its hosts is clearly a very fertile topic for future research.

Section 4. Future work

Research programmes continuing from the work described in this thesis would involve the following:-

(1) A study of the anatomical and morphological changes in the petioles of *Pelargonium zonale* resulting from *Cuscuta* attack, paying particular attention to the swelling and the formation of any physical barriers to the attack of the parasite.

(2) A more intensive study of the activities and localisation of cell wall degrading enzymes in relation to the penetration process. In particular the role of PGURASE in the interaction must be resolved. Antibodies raised to the prominent enzymes would be essential tools for the task of localising their origin and sites of action. Positive enzyme localisation at the interface between the host and parasite would for example strongly suggest that cell wall fragments are generated.

(3) An investigation to determine whether cell wall fragments are released in the region of the host/parasite interface. Probably a method involving radioactive labelling of host or parasite would have to be developed. The amounts of wall fragment might be for example compared in infected and uninfected swollen and unswollen petioles.

(4) An investigation into the response of *Pelargonium* petioles to wounding and to the application of cell wall fragments generated using purified *Cuscuta* enzymes on isolated Pelargonium cell walls.

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Appendices

Appendix 2.6.1 The table equilavents for the flow times of the reaction mixtures in the viscometric assays of cellulase activity(see section 6.1 of Ch2)

t	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
45	4.81	4.80	4.79	4.78	4.76	4.75	4.74	4.73	4.72	4.71
44	4.93	4.92	4.91	4.89	4.88	4.87	4.86	4.85	4.83	4.82
43	5.06	5.04	5.03	5.02	5.01	4.99	4.98	4.97	4.95	4.94
42	5.19	5.18	5.16	5.15	5.14	5.12	5.11	5.09	5.08	5.07
41	5.32	5.32	5.30	5.29	5.27	5.25	5.24	5.23	5.22	5.20
40	5.48	5.47	5.45	5.43	5.42	5.40	5.39	5.38	5.36	5.35
39	5.64	5.62	5.61	5.59	5.57	5.56	5.54	5.54	5.51	5.50
38	5.80	5.79	5.77	5.75	5.74	5.72	5.70	5.69	5.67	5.65
37	5.98	5.97	5.95	5.93	5.91	5.89	5.88	5.86	5.84	5.82
36	6.17	6.15	6.13	6.11	6.10	6.07	6.06	6.04	6.02	6.00
35	6.37	6.36	6.33	6.31	6.29	6.27	6.25	6.23	6.21	6.19
34	6.59	6.57	6.55	6.53	6.50	6.48	6.46	6.44	6.42	6.40
33	6.82	6.80	6.78	6.76	6.74	6.71	6.68	6.66	6.64	6.62
32	7.07	7.06	7.02	6.98	6.98	6.95	6.95	6.91	6.87	6.87
31	7.34	7.31	7.31	7.27	7.23	7.23	7.19	7.19	7.15	7.10
30	7.62	7.61	7.57	7.57	7.52	7.48	7.48	7.44	7.40	7.40
29	7.98	7.93	7.88	7.88	7.84	7.79	7.79	7.75	7.70	7.66
28	8.27	8.26	8.22	8.22	8.17	8.12	8.12	8.07	8.02	7.98
27	8.67	8.62	8.62	8.57	8.51	8.46	8.46	8.41	8.36	8.36
26	9.05	9.04	8.99	8.93	8.93	8.88	8.83	8.77	8.77	8.72
25	9.50	9.50	9.44	9.38	9.32	9.32	9.27	9.21	9.15	9.10
24	9.99	9.98	9.92	9.86	9.80	9.80	9.74	9.68	9.62	9.56
23	10.57	10.50	10.43	10.43	10.36	10.30	10.23	10.17	10.11	10.04
22	11.27	11.12	11.05	10.98	10.91	10.84	10.80	10.77	10.70	10.63
21	11.79	11.79	11.71	11.63	11.56	11.48	11.41	11.41	11.33	11.26
20	12.59	12.51	12.42	12.42	12.34	12.26	12.18	12.10	12.02	11.94

Appendix 2.6.1 Continued

t	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
19	13.47	13.37	13.28	13.19	13.11	13.02	12.93	12.84	12.76	12.67
18	14.42	14.42	14.32	14.22	14.03	13.93	13.84	13.74	13.65	13.56
17	15.57	15.46	15.35	15.24	15.14	15.03	14.93	14.82	14.72	14.62
16	16.97	16.84	16.72	16.60	16.36	16.25	16.13	16.02	15.90	15.79
15	18.52	18.38	18.24	17.98	17.85	17.72	17.59	17.46	17.21	17.08
14	20.26	20.26	19.95	19.80	19.65	19.36	19.22	19.07	18.93	18.65
13	22.56	22.38	22.21	21.87	21.70	21.53	21.20	21.04	20.88	20.57
12	25.41	25.21	24.81	24.61	24.22	24.03	23.65	23.47	23.10	22.90
11	28.98	28.50	28.27	27.80	27.35	27.12	26.68	26.47	26.04	25.83
10	33.50	32.92	32.36	32.08	31.54	31.00	30.48	30.22	29.72	29.47
9	39.31	38.95	37.91	37.57	36.90	36.26	35.62	34.99	34.39	34.08
8	47.81	46.91	45.59	45.16	43.91	43.50	42.30	41.91	40.77	40.70
7	60.02	58.81	57.05	55.92	54.27	53.27	51.67	51.16	49.69	48.77
6	78.16	76.47	74.02	72.45	70.16	68.69	65.86	64.50	62.53	61.06
5	111.64	107.69	102.69	99.13	95.73	92.48	89.36	87.36	83.52	81.69
4	177.31	167.94	159.20	151.00	143.3	137.81	131.08	126.23	120.12	115.78

Appendix 2.6.2 The table equivalents for the flow times of reaction mixtures during viscometric assays to measure polygalacturonase activity (see section 6.2 Ch2)

t	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
5	5.380	5.260	5.140	5.040	4.90	4.857	4.766	4.685	4.610	4.537
6	4.470	4.406	4.345	4.287	4.232	4.180	4.129	4.082	4.036	3.992
7	3.950	3.910	3.871	3.833	3.798	3.763	3.729	3.697	3.666	3.636
8	3.607	3.579	3.551	3.525	3.499	3.474	3.450	3.426	3.403	3.381
9	3.360	3.339	3.318	3.298	3.279	3.260	3.241	3.223	3.205	3.188
10	3.171	3.155	1.139	3.123	3.108	3.092	3.078	3.063	3.049	3.035
11	3.022	3.008	2.997	2.982	2.970	2.958	2.946	2.934	2.922	2.911
12	2.899	2.888	2.877	2.867	2.856	2.846	2.836	2.826	2.816	2.806
13	2.797	2.788	2.778	2.769	2.760	2.752	2.743	2.734	2.726	2.718
14	2.709	2.701	2.693	2.686	2.6789	2.670	2.663	2.655	2.648	2.641
15	2.634	2.627	2.620	2.613	2.606	2.600	2.593	2.586	2.580	2.574
16	2.567	2.561	2.555	2.549	2.543	2.537	2.531	2.526	2.520	2.514
17	2.509	2.503	2.498	2.492	2.487	2.482	2.476	2.471	2.466	2.461
18	2.456	2.451	2.446	2.441	2.436	2.432	2.427	2.422	2.418	2.413
19	2.408	2.404	2.400	2.395	2.391	2.386	2.382	2.378	2.374	2.370
20	2.365	2.362	2.353	2.353	2.349	2.345	2.341	2.337	2.333	2.330
21	2.326	2.322	2.319	2.316	2.311	2.308	2.304	2.300	2.297	2.293
22	2.290	2.287	2.283	2.280	2.276	2.273	2.270	2.266	2.263	2.260
23	2.257	2.253	2.250	2.247	2.244	2.241	2.238	2.235	2.232	2.229
24	2.226	2.228	2.220	2.217	2.214	2.211	2.208	2.205	2.203	2.200
25	2.197	2.194	2.192	2.189	2.186	2.183	2.181	2.178	2.176	2.173
26	2.170	2.168	2.165	2.163	2.160	2.158	2.155	2.153	2.150	2.148
27	2.145	2.143	2.140	2.138	2.136	2.133	2.131	2.129	2.126	2.124
28	2.122	2.119	2.117	2.115	2.113	2.110	2.108	2.106	2.104	2.102
29	2.100	2.097	2.095	2.093	2.091	2.089	2.087	2.085	2.083	2.081
30	2.079	2.077	2.075	2.073	2.071	2.069	2.067	2.065	2.063	2.061
31	2.059	2.057	2.055	2.053	2.051	2.049	2.048	2.046	2.044	2.042
32	2.040	2.038	2.036	2.034	2.033	2.031	2.029	2.027	2.026	2.024
33	2.022	2.020	2.019	2.017	2.015	2.014	2.012	2.010	2.009	2.007
34	2.005	2.003	2.002	2.000	1.998	1.997	1.995	1.993	1.992	1.990
35	1.989	1.988	1.987	1.985	1.983	1.982	1.981	1.979	1.977	1.976

Appendix 3.3.3.3 The absorbance values for standard galacturonic acid solutions see section 3.3 of Ch3

conc of G.A. in mg ml^{-1}	Absorbance at 520nm 1	2	3	mean absorbance
0.1	0.203	0.199	0.194	0.199
0.2	0.456	0.489	0.468	0.471
0.3	0.679	0.703	0.743	0.707
0.4	0.914	0.908	0.905	0.909
0.5	1.126	1.124	0.129	1.126

Appendix 4.I.2.4 The response of *Cuscuta* shoot tips to touch 0.5-4.0 cm from the apex

The aim of the experiment was to see if shoot tips were sensitive to touch 0.5-4.0 cm from the apex.

Experimental A rectangular tank was prepared by filling it full of water. Rubber bands were stretched across the parallel sides forming a net-like pattern above the surface of the water (see Fig 4.I.2.4a) The intersection of four bands formed an adjustable aperture (see Fig 4.I.2.4b) *Cuscuta* shoot tips were held at these intersections with the ends trailing in the water. The shoot tips were held at a pre-arranged distance from the shoot tip. Five shoot tips were held at 0.5, I.0, 2.0, 3.0 and 4.0 cm from the apex. They were then left under greenhouse conditions for 24hr to see how the shoot tips responded to being held with the rubber bands.

The response of the shoot tips

None of the shoot tips changed their pattern of growth as a result of being held by the bands. However when the apices of shoot tips made contact with other shoot tips after two or more further days, they rapidly formed tight coils around each other.

This experiment showed that coiling could not be stimulated by exerting a collar of pressure at 0.5-4.0 cm from the shoot apex.

Fig 4.1.2.4a The net-like pattern of rubber bands across the surface of a tank of water

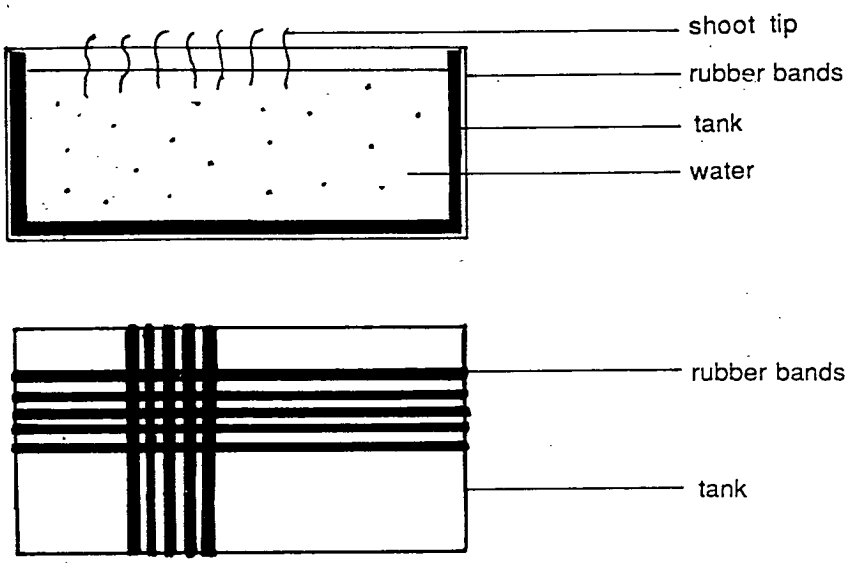
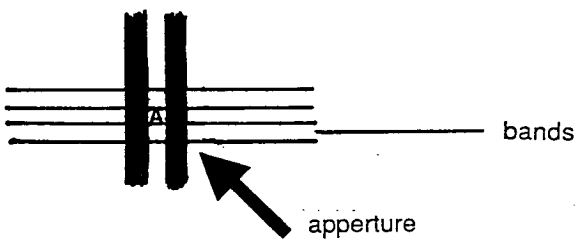


Fig 4.1.2.4b The adjustable apperture formed by intersecting rubber bands



Appendix 4.2.3.I

A table showing the difference in the protein extracted from *Cuscuta* by the freeze/thaw method and the maseration method. 30µl samples of extract were assayed for protein using the Beardens assay procedure described in ch2. (FT) = freeze/thaw, (MS) = maceration

Sample	Treatment	Protein per sample (µg)	Mean	se
1	FT	19.08		
2	FT	22.83		
3	FT	23.95		
4	FT	23.35	20.97	+ 1.36
5	FT	24.59		
6	FT	17.68		
7	FT	15.30		
8	MS	25.89		
9	MS	51.03		
10	MS	34.49		
11	MS	-	35.34	+ 3.84
12	MS	28.05		
13	MS	41.35		
14	MS	31.24		

Appendix 4.2.3.8

This table shows the reducing power generated by the enzyme extract from tomato mixed with a solution of polygalacturonic acid and incubated for 4 hr. Reaction mixtures consisted of enzyme extraction 0.05M acetate buffer pH 4.5 polygalacturonic acid, 0.05M acetate buffer pH 4.5 and sodium dithionite (0.1mM). One set of reaction mixtures were incubated for 4 hours before their reducing power was estimated the others had their reducing power measured immediately. A higher absorbance value reflects a higher reducing power.

Sample	Treatment	Absorbance
1	Incubated	1.357
2	Unincubated	0.420

These values show polygalacturonase was present in extracts of ripe tomato.